Cellular Innate Immune Responses to Lung Resection via Video-Assisted Thoracoscopic Surgery (VATS) and Thoracotomy: Predictors of Post-Operative Pneumonia

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ABSTRACT

Background and Objectives: The pathophysiology of post-operative pneumonia following lung resection is poorly understood despite it being the most common complication which may lead to death. The role of the acute inflammatory response following lung resection, in particular innate immune cells, was investigated and used to identify biomarkers for post-operative pneumonia. Comparison of inflammatory responses to resection undertaken by video-assisted thorascopic surgery (VATS) and thoracotomy was also evaluated.

Methods: Patients undergoing lung resection for suspected bronchogenic carcinoma were recruited. Objective pre-defined criteria were used to diagnose pneumonia. Bronchoalveolar lavage (BAL) was conducted in the contra-lateral lung pre- and post-operatively to measure cellular composition and cytokines. Blood was sampled pre-operatively and 6-, 24- and 48-hours post-operatively primarily to assess neutrophil phagocytic capacity, monocyte subsets, monocyte cytokine responses to lipopolysaccharide (LPS) stimulation and serum cytokine responses. Exhaled nitric oxide (eNO) was also measured at these time points. Patient groups were compared using paired or student t-tests together with ANOVA/ANCOVA modelling. The predictive strength of the biomarkers identified was tested.

Results: 40 patients were recruited. 26 patients (65%) underwent major lung resection using VATS and 14 (35%) thoracotomy. There was a post-operative blood monocytosis ($p<0.0005$) with an absolute expansion of classical and intermediate monocytes ($p=0.001$) and a relative fall in non-classical monocytes ($p<0.005$). Post-operatively blood monocytes became more pro-inflammatory with an overall significant increase in IL-8 ($p=0.034$) and TNF-α ($p=0.028$) together with an increase in IL-6 ($p=0.028$) and IL-10 by 48 hours ($p=0.010$). VATS was associated with a smaller release of IL-10 only ($p=0.011$). There was a general trend towards post-operative reduction in neutrophil phagocytosis of zymosan (in suspension) on ANOVA modelling ($p=0.047$). Lung

resection led to an increase in serum cytokines IL-6, IL-8 and IL-10 which peaked at 24hrs before falling ($p<0.0005$). ANOVA modelling confirmed significantly lower levels of serum cytokines in VATS patients compared with thoracotomy ($p=0.026$ for IL-6, $p=0.018$ for IL-8 $p=0.047$ for IL-10). No significant post-operative change was found for IL-1β, TNF-α and IL-12p70 ($p>0.05$). Bronchoalveolar lavage fluid (BALF) and blood samples demonstrated a relative post-operative leucocytosis due principally to neutrophilia. A relative blood lymphopenia and thrombocytopenia developed post-operatively ($p<0.0005$). VATS was associated with a lower fall in serum albumin ($p=0.001$). BALF from the non-operated lung became more pro-inflammatory immediately post-operatively with an increase in IL-6 ($p<0.0005$), IL-8 ($p=0.017$), IL-10 ($p=0.018$) and IL-1β ($p=0.002$). eNO tended to fall post-operatively which reached significance at 48 hrs ($p=0.029$).

14 patients developed pneumonia. Pre-operatively, a blood neutrophil count above $5.04x10^9$/L had a relative risk (RR) for pneumonia of 3.3 (95% confidence interval (CI_{95}) 1.1-10.1), and a BAL cell count of greater than $1.04x10^5$/ml had a RR of 3.4 (CI_{95} 1.3-9.0), whilst LPS-stimulated monocyte secretion of IL-12 of less than 0.15 pg/ml/μg protein had a RR of 3.0 (CI_{95} 1.2-7.3). At 24 hours post-operatively, LPS-stimulated release from monocytes of IL-10 greater than 1.99 pg/ml/μg protein (RR 4.1, CI_{95} 1.3-12.3) and IL-6 greater than 414 pg/ml/μg protein (RR 3.1, CI_{95} 1.2-8.1) were predictive of pneumonia.

Conclusion: Lung resection is associated with significant early pro- and anti-inflammatory responses. VATS resection invoked significantly lower levels of serum cytokines and albumin changes compared with thoracotomy suggesting VATS lobectomy should be the surgical treatment strategy of choice for early stage lung cancer. No difference in neutrophil function or monocyte function was however observed between the surgical groups. Clinical benefits of this reduced inflammation need to be evaluated in a larger cohort of patients. Relative pre-operative leucocytosis in

blood and BAL together with monocyte hyper-responsiveness in the early post-operative period is associated with the development of pneumonia. These findings warrant further investigation for their predictive power in accurately identifying post-operative pneumonia. Ultimately, they may be incorporated into a risk stratification model enabling targeted prophylactic or earlier therapeutic intervention.

DECLARATION

"I declare that:

1. This thesis has been composed by myself;
2. The work is entirely my own unless otherwise specified;
3. The work has not been submitted for any other degree or professional qualification".

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LIST OF ABBREVIATIONS

ALI	Acute Lung Injury
ANCOVA	Analysis of covariance (statistical analysis)
ANOVA	Analysis of Variance (statistical analysis)
ARDS	Acute Respiratory Distress Syndrome
ASA	American Society of Anaesthesiologists
ATS	American Thoracic Society
BAL	Bronchoalveolar lavage
BALF	Bronchoalveolar lavage fluid
BD	Becton Dickinson
BMI	Body Mass Index
BSA	Bovine Serum Albumin
BTS	British Thoracic Society
CARS	Compensatory anti-inflammatory response syndrome
CI_{95}	95% Confidence Interval

COPD	Chronic obstructive pulmonary disease
CRP	C-reactive protein
CSO	Chief Scientist Office
CXR	Chest X-Ray (Chest roentogram)
DLCO	Diffusing capacity of the lung for carbon monoxide
eNO	Exhaled nitric oxide
ERS	European Respiratory Society
FENO	Fractional Exhaled Nitric Oxide
FEV_1	Forced Expiratory Volume in one second
FFC	Forward Scatter
FNA-EBUS	Fine Needle Aspiration-Endobronchial Ultrasound
FVC	Forced Vital Capacity
GRO-α	Growth regulated peptide alpha
Hb	Haemoglobin
HLA-DR	Human leucocyte antigen DR

IL	Interleukin
IL-1RA	Interleukin-1 receptor antagonist
IMDM	Iscove's Modified Dulbecco's Medium
IQR	Interquartile Range
iNOS	Inducible nitric oxide synthase
KCO	Diffusing capacity of the lung for carbon monoxide per unit volume of lung
LRTI	Lower Respiratory Tract Infection
MHC II	Major Histocompatibility Complex Class II
MS	Mediastinoscopy
MSSU	Mid-stream specimen of urine
NHS	National Health Service
NICE	National Institute of Clinical Excellence
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
NSCLC	Non-small cell lung cancer

OLV	One lung ventilation
PBS	Phosphate Buffered Saline
PPB	Parts per billion
PET-CT	Positron Emission Tomography-Computed Tomography
PFTs	Pulmonary Function Tests
RR	Relative Risk
SIRS	Systemic Inflammatory Response Syndrome
SSC	Side Scatter
TCO	Transfer factor for carbon monoxide
TNF-α	Tumour necrosis factor alpha
UK	United Kingdom
VATS	Video Assisted Thoracoscopic Surgery
WBC	White Blood Cell (Leucocyte)

I - INTRODUCTION

Despite the overall incidence of lung cancer falling in recent decades in the United Kingdom (UK), following the decline in smoking, it remains the second commonest cancer diagnosed in men after prostate cancer and third in women after breast and colorectal cancer (Cancer Research UK, 2009). In 2009 there were 41,428 new cases diagnosed in the UK, 56% of which were in men. However, in Scotland alone lung cancer remains the most commonly diagnosed cancer. Indeed, the incidence in Scotland is amongst the highest in the world, a position attributed to previous industrial asbestos exposure and the remaining high prevalence of smoking. Lung cancer continues to be the leading cause of cancer death in the UK accounting for 24% of all male cancer deaths and 21% of all female deaths in 2010 (Cancer Research UK, 2010). It is also the leading cause of cancer death worldwide (Jemel et al., 2011).

Lobectomy is the optimal curative strategy for patients with stage I and II non-small cell lung cancer (NSCLC) (NICE Clinical Guideline 121, 2011). It confers a five-year survival benefit, which for stage I NSCLC is in the region of 78% (Walker et al., 2003). Traditionally lung resection has involved a large incision via a thoracotomy with exposure gained through the insertion of a retractor between rib spaces and rib spreading. Since the early 1990s minimally invasive techniques have been developed for lobectomy via video-assisted thoracoscopic surgery (VATS) (Walker, 1999). This avoids rib spreading thereby reducing post-operative pain and is associated with faster post-operative recovery together with a reduced post-operative inflammatory response (Nagahiro et al., 2001 and Craig SR et al., 2001).

Although peri-operative mortality and morbidity have fallen in recent decades (Ferguson et al., 2008), major lung resection, via VATS or thoracotomy, continues to represent a significant risk for the patient. The UK national average in-patient mortality rate for malignant lung resection is in the order of 2% for lobectomy/bilobectomy and 6.5% for pneumonectomy (SCTS, 2010). The most common complication post-

operatively is lower respiratory tract infection (LRTI). The exact incidence varies considerably between surgical series, both prospective and retrospective, with values ranging from 2-40% (Schussler et al., 2006). The variable incidence probably reflects the characteristics of the patients, extent of resection, antibiotic prophylaxis and patient management. Moreover, the criteria used to define post-operative pneumonia vary considerably between surgical series.

Post-operative pneumonia is associated with an increased length and cost of hospital stay and may lead to respiratory failure, death or the development of acute lung injury (ALI) or the more severe form acute respiratory distress syndrome (ARDS) (Uramato et al., 2001 and Baudouin, 2003). Both ALI and ARDS are characterised by bilateral pulmonary infiltrates in the absence of cardiac failure with severe hypoxia (Bernard et al., 1994).

Clinical Risk Factors for Post-Operative Pneumonia

Surprisingly little is understood about the aetiology of pneumonia following lung resection. A number of retrospective clinical studies have been published identifying risk factors for post-operative pneumonia. One of the earliest retrospective papers identified the diffusing capacity of the lung for carbon monoxide (DLCO) to predict mortality and was the sole predictor of pulmonary complications out of 38 pre-operative and intra-operative risk factors examined (Ferguson et al., 1988). A more recent study by the same group identified post-operative predicted DLCO to be the single strongest predictor of pulmonary morbidity and mortality in patients with and without chronic obstructive pulmonary disease (COPD) (Ferguson et al., 2008). However, a recent study concluded that DLCO and PFTs (Pulmonary function tests) in general have no role in determining pulmonary complications after VATS lobectomy (Berry et al., 2010).

One further study of 242 patients undergoing pneumonectomy for lung cancer sought to identify independent risk factors for the development of pulmonary complications

(Algar et al., 2003). Univariate analysis identified increased age, COPD, heart disease, no previous record of chest physiotherapy, poor post-operative predicted forced expiratory volume in one second (FEV_1) and prolonged anaesthetic time as independent risk factors for pulmonary complications. In a multiple logistic regression model, prolonged anaesthetic time, poor post-operative predicted FEV_1, heart disease, no previous chest physiotherapy and COPD were found to be independent risk factors for pulmonary complications.

A recent retrospective study utilising The Society of Thoracic Surgeons General Thoracic Surgery Database identified predictors of death and major morbidity in 1,267 patients undergoing pneumonectomy (Shapiro et al., 2010). Age >65, male sex, congestive cardiac failure, FEV_1 <60% predicted, benign lung disease and extra-pleural pneumonectomy were independent risk factors for major morbidity. Chemoradiotherapy was also a risk factor in lung cancer patients.

Indeed, a number of scoring systems have been published in order to predict in-hospital mortality following general thoracic surgery based on clinical variables (Berrisford et al., 2005 and Falcoz et al., 2007). The system of Falcoz et al., termed 'Thoracoscore', is gaining some acceptance and is currently recommended in the British Thoracic Society (BTS) guidelines for pre-operative assessment in lung cancer patients (Lim et al., 2010). Age, gender, American Society of Anaesthesiologists (ASA) Score, performance status, dyspnoea score, priorty of surgery, procedure class, diagnostic group and co-morbidity are used to predict in-hospital mortality for all thoracic surgery procedures. The accuracy and usefulness of the scoring has however been questioned by a recent paper in patients following lung resection (Bradley et al., 2012). Further, many of these clinical risk factors are fixed and not suitable for prophylactic or therapeutic intervention.

Bacterial Colonisation

One focus of basic scientific research has been airway colonisation with pathogenic microorganisms and the development of post-operative pneumonia. It is well established that up to a third of smokers and patients with COPD have bacterial colonisation of the lower airways with potentially pathogenic microorganisms (Soler et al., 1999). Colonisation patterns do not differ markedly in lung cancer patients from COPD patients, many of whom have COPD (D'Journo et al., 2011). However, in the specific instance of an obstructing bronchial carcinoma, Gram negative bacilli and anaerobes may be found distal to the obstruction (Liaw et al, 1994). Upper respiratory tract colonisation has been shown to be significantly higher in lung cancer patients (59.1%) compared to patients without malignancy (37.3%) and healthy volunteers (37.8%) (Hirakata et al., 1997).

Despite the undoubted airway colonisation in a significant proportion of lung cancer patients, associating this with post-operative infection has been controversial. A number of studies have concluded that airway colonisation is a significant independent risk factor for post-operative pneumonia (Wansbrough-Jones et al., 1991 and Schussler et al., 2006). Others could find no link (Ioanas et al, 2002). A meta-analysis on the issue was recently published involving 7 prospective studies including 1083 patients and concluded there was reasonable evidence for a link (D'Journo et al., 2011). Matching the actual microbiological organisms found pre- and post-operatively is also inconsistent. Some of the discrepancy may relate to sampling methods for microbiology which cannot represent all the distal airways of a particular patient and may miss specific areas of colonisation. Thus, while it is apparent that there is an association between bacterial colonisation of lower airways and post-operative infection the link is relatively weak.

Acute Inflammation

The clinical and microbiological factors mentioned above together with post-operative pain requiring opiate analgesia, poor cough and failure to clear airway secretions may predispose patients to infection. Pre-operative nutritional status has also been linked to post-operative complications (Jagoe et al., 2001). All of these factors however need to be seen in the context of an underlying acute inflammatory response to the trauma of surgery.

One of the earliest studies linking inflammation and post-operative outcome reported the use of C-reactive protein (CRP) in predicting post-pneumonectomy empyema in a cohort of 151 patients (Icard et al., 1993). Amar and colleagues examined a number of markers of inflammation peri-operatively and related these to major post-operative complications, not just respiratory, in 153 patients undergoing lung resection or exploratory thoracotomy alone (Amar et al., 2007). A strong association was found for high pre-operative serum CRP (area under the curve (AUC) 0.86), low albumin (AUC 0.86) and high interleukin-6 (IL)-6 (AUC 0.79)). This suggested pre-operative sub-clinical inflammation is likely to be important in the pathogenesis of major post-operative complications.

Further, the individual patient inflammatory response to the trauma of surgery may predict for post-operative complications. Shaw et al. examined polymorphisms in key inflammatory alleles, IL-6 and TNF-α, in 155 patients (Shaw et al., 2005). They found that pro-inflammatory genotypes predicted for post-operative complications, particularly if homozygous for the alleles. In a multi-variate analysis, they found that cardiovascular disease, IL-6 genotype and TNF-α genotype were independently predictive of complications, with an area under the curve of 0.765 for the entire model. Although serum levels of the cytokines were not measured peri-operatively this was an intriguing study into inflammation as a cause of post-operative morbidity in thoracic surgery patients.

Indeed, profound acute inflammation may lead to a systemic inflammatory response syndrome (SIRS) that can result in organ dysfunction and ultimately death (Bone, 1996). SIRS however also invokes a counter response termed compensatory anti-inflammatory response syndrome (CARS) in order to restore homeostasis. Imbalance between these two responses may be harmful. Prevalence of CARS may lead to immune anergy and vulnerability to infection.

Neutrophils are the key cell type in the acute inflammatory response. They are rapidly recruited to sites of infection and tissue injury where they phagocytose and destroy invading pathogens. Studies in non-surgical critically-ill patients have demonstrated dysfunction of peripheral blood neutrophil phagocytosis (Simms et al., 1994 and Kaufmann et al., 2006). Subsequent work has shown this to be related to complement activation, specifically complement factor 5a (C5a), which inhibited RhoA activation preventing actin polymerisation (Conway Morris 2009 and 2011). From a cohort of 60 critically-ill patients, C5a-mediated neutrophil dysfunction was a strong predictor of nosocomial infection. Whether neutrophil dysfunction occurs after lung resection, and its relation to post-operative complications, has not been studied to date.

Another key cell in the innate immune response is the monocyte. Monocytes circulate in the peripheral blood for a few days after leaving the bone marrow before migrating into tissue to eventually become dendritic cells or macrophages. Their role includes immune defense and tissue repair (Ziegler-Heitbrock et al., 2010). They produce a range of cytokines, activate T and B lymphocytes and process and present antigen (Ziegler-Heitbrock, 2007). Three main subsets of monocyte have been identified in humans, that is, classical ($CD14^{++}$, $CD16^{-}$), intermediate ($CD14^{++}$, $CD16^{+}$) and non-classical ($CD14^{+}$, $CD16^{++}$). Classical monocytes form the majority of circulating monocytes with non-classical monocytes only representing up to 20% of the circulating pool. Intermediate monocytes are thought to be a precursor to non-classical cells in that their pool expands in infection before expansion occurs in the non-classical pool.

Monocyte deactivation has been described in sepsis and is associated with a much higher mortality (Döcke et al., 1997). This deactivation is characterised by markedly reduced HLA-DR expression with a reduced antigen-presenting capacity and a profound reduction of the ability to produce lipopolysaccharide (LPS)-induced tumour necrosis factor alpha (TNF-α) in vitro. Monocyte counts and function have been analysed following major cardiac surgery involving use of the extra-corporeal cardiopulmonary bypass circuit (Franke et al., 2009). A monocytosis was reported on day one but the monocyte function was impaired, demonstrated by a reduced capacity to synthesise IL-12 and TNF-α, and lower HLA-DR expression. Cytokine-producing capacity returned to normal on day three but the suppression of HLA-DR persisted. Similar monocyte immunosuppression has been found in trauma patients admitted to a trauma centre, the duration of which reflected the severity of trauma sustained (Wutzler et al., 2009). Few studies have been published on monocyte changes post-lung resection. Nosotti et al. found lower HLA-DR expression in lung cancer patients pre-operatively and short-term changes in subsets after lobectomy via thoracotomy which returned to normal on day 30. However, the first post-operative sample was taken on day 5 missing many of the potentially key inflammatory changes. Moreover, actual monocyte function was not assessed, nor were the classical markers defining monocyte subsets.

Inflammatory changes at a local pulmonary level are poorly characterised after lung resection with scarce studies published. One-lung ventilation (OLV) of the non-operated lung is mandatory during lung resection. This may lead to a form of lung injury in itself through a number of proposed mechanisms (Baudouin, 2003). During collapse of the operated lung, blood flow is reduced which may lead to a form of ischaemia-reperfusion injury once dual lung ventilation is resumed. The consequent increased flow of blood to the contralateral lung intra-operatively may lead to pulmonary capillary stress and may produce capillary injury. The contralateral lung may

also suffer from ventilator-induced lung injury depending on tidal volumes used in addition to hyperoxia and the generation of reactive oxygen species (ROS).

Bronchoalveolar lavage has rarely been used to characterise these inflammatory processes after lung resection. One study involved 15 patients (Breunig et al., 2011). IL-6, IL-1 receptor antagonist (IL-1 RA) and growth-regulated peptide alpha (GRO-α) were measured after induction of anaesthesia and after lung resection in BALF from both lungs as well as pleural fluid and blood. A significant increase in IL-6 and IL-1 RA was found but not GRO-α in both lungs after surgery. This suggests the initiation of both pro- and anti-inflammatory responses at the time of surgery. IL-6 was higher in the operated lung post-operatively but no difference was observed between the lungs for IL-1RA and GRO-α.

More information on the impact of OLV during surgery can be gleaned from oesophagectomy patients. Cree et al. showed higher levels of IL-8 in BALF than peripheral blood in 21 patients but no difference between collapsed and ventilated lungs (Cree et al., 2004). Importantly however the collapsed and ventilated samples were taken from two different groups of patients. Zingg et al. found higher levels of IL-6, IL-8 and IL-1 RA in the ventilated lung prior to extubation when conducting BAL in both lungs of the same patient (Zingg et al., 2010). Clearly, inflammatory changes occur in the ventilated, comparatively healthy lung following OLV which is of considerable interest to evaluate further for lung resection patients through BAL.

Nitric oxide is a diatomic molecule formed from oxygen and nitrogen. It is a soluble gas containing an unpaired electron, making it highly reactive. It has a short half-life and is able to freely move across cell membranes without specific transporters (Masri F, 2010). It is produced throughout the body at three principal sites; the macrophage, the airway and vascular endothelium. It is produced by three isoforms of nitric oxide synthase (NOS); inducible NOS (NOS2), neuronal (NOS1) and endothelial (NOS3) (Kobzik L, 2009). Nitric oxide production may be increased by upregulation of

inducible nitric oxide synthase (iNOS). Its role is complex but the balance of evidence suggests that it plays a role in innate immune defense in humans. It is also a vaso- and bronchodilator and contributes to co-ordination of ventilation/perfusion (Marczin et al., 1997). Its vasodilator properties on endothelium are the principal reason for inhaled nitric oxide use in managing established ARDS and persistent pulmonary hypertension of neonates (Adler et al., 2009).

Several studies have demonstrated increased levels of nitric oxide in the exhaled breath of patients with inflammatory lung diseases. Exhaled nitric oxide levels are increased in asthmatic patients, whom suffer a chronic eosinophilic inflammatory process, which decrease with anti-inflammatory therapy such as corticosteroids (Barnes et al., 1995 and Yates et al., 1995). Conversely, in established ARDS, levels of exhaled nitric oxide are reduced (Brett et al., 1998) as well as following lung transplantation (Marczin et al., 1997). The possible reason for this reduction is down regulation of constitutive or type I nitric oxide synthase (NOS) and an inability to produce type II NOS or iNOS. Further, eNO may react with highly reactive molecules such as superoxide forming peroxynitrite.

Marczin et al. noted that no change in exhaled nitric oxide was associated with a good clinical outcome whereas a reduction was associated with morbidity and mortality after transplantation surgery in a small cohort of patients (Marczin et al., 1997). Conversely, increased levels of eNO after haematopoietic stem cell transplantation appear to be a marker for pulmonary complications in children (Fazekas et al., 2012). Few studies have examined exhaled nitric oxide peri-operatively after lung resection. Moreover, by using variable flow rates of exhaled breath to measure exhaled nitric oxide, alveolar and upper airway contributions to total exhaled nitric oxide can be calculated using the two-compartment model of airway dynamics (Pietropaoli et al., 1999).

Hypotheses

This study sought to determine whether respiratory complications after lung resection for suspected lung cancer could be related to peri-operative changes in innate immune function. Two fundamental hypotheses were proposed:-

(i) An exaggerated local pulmonary and systemic inflammatory response early in the course of surgery predicts for respiratory complications such as pneumonia;
(ii) Impaired cellular innate immune responses may predispose patients to acquiring post-operative pneumonia.

Study Aims

In order to test these hypotheses an in-depth study of pulmonary and systemic innate immune responses following lung resection for lung cancer was devised relating these changes to the type of surgery undertaken and post-operative complications. Such a comprehensive study has not been undertaken before. The specific aims were:-

(i) To determine baseline and post-operative changes in pulmonary cytokines, total protein and differential cell count using bronchoalveolar lavage from the comparatively healthy, non-operated lung;
(ii) To measure baseline and serial trends in exhaled nitric oxide;
(iii) To determine baseline and serial changes in neutrophil phagocytic capacity;
(iv) To evaluate baseline and serial changes in monocyte subsets and monocyte cytokine responsiveness to LPS stimulation;
(v) To determine baseline and post-operative trends in leucocyte and differential cell count;
(vi) To compare baseline innate immunity and inflammatory measurements of pre-operative lung cancer resection patients with that of a group of healthy control patients of a similar age and mixed gender;

(vii) To compare innate immunity and inflammatory responses of patients undergoing lung resection via VATS and thoracotomy;

(viii) To accurately determine the rate of post-operative pneumonia, acute lung injury and acute respiratory distress syndrome using prospectively defined criteria;

(ix) To establish which of the above biological indices best predict for the development of post-operative pulmonary complications after major lung resection for lung cancer.

II - METHODOLOGY

Financial Funding

The project was funded by a Scottish Chief Scientist Office (CSO) research grant (CZB/4/604). Additional funding was obtained from The Royal Infirmary of Edinburgh, Thoracic Surgery Unit Charitable Funds.

Ethical and Institutional Approval

The study and protocol were approved by The Lothian Research Ethics Committee (LREC No: 09/S1101/11) and NHS Lothian Research and Development (ID No: 2009/R/RES/03).

Patient Recruitment

Patients referred for Thoracic Surgery at The Royal Infirmary of Edinburgh with proven or clinically suspected lung cancer were eligible for this study. The Edinburgh Thoracic Surgery unit is a tertiary referral centre and one of the largest units in the UK. All patients referred for surgical resection in Edinburgh undergo bronchoscopy and cervical mediastinoscopy routinely before being offered surgical resection. The aim is to identify N2 disease which, by definition, is incurable by surgery alone (Nice Clinical Guideline 121) and may not have been identified on staging investigations such as Positron Emission Tomography-Computed Tomography (PET-CT) or Fine Needle Aspiration-Endobronchial Ultrasound (FNA-EBUS) (Carnochan et al., 2009).

It was ahead of surgical staging that patients under the care of a single Consultant Cardiothoracic Surgeon, Mr. William S. Walker, were approached to take part in the study. Participation was entirely voluntary with no financial incentive offered. Written informed consent was obtained from interested patients and a confidential record kept

of their personal details. Patients were excluded from the study if there was evidence of metastatic disease on routine pre-operative staging, if mediastinoscopy staging revealed tumour involvement in lymph nodes or at any point on the request of the patient or Consultant Surgeon. A total of 53 patients were eligible and consented to take part in the study however 13 patients were excluded leaving a total of 40 patients in the study. The reasons for exclusion were that 7 patients had tumour positive mediastinoscopy, 2 patients withdrew post-operatively as they did not wish to undergo the tests required, 1 patient was excluded because pneumonectomy was required prior to ethical approval for inclusion of pneumonectomy patients being obtained, 1 patient was deemed unfit for resection after staging bronchoscopy and mediastinoscopy and 2 patients were excluded due to the scheduling of lung resection surgery and the inability to process more than 2 patient resections per week.

Control Patient Recruitment

Ten patients of mixed gender but a similar age range to the study population were recruited to act as a control group for the blood and exhaled nitric oxide tests. They were identified with the co-operation of Dr. Hamish Reid, General Practitioner, from the Penicuik Health Centre patient database and invited to take part in the research study. Each patient voluntarily attended The Royal Infirmary of Edinburgh Thoracic Surgery ward for this purpose where written, informed consent was obtained. Patients were eligible if they were considered generally healthy without significant illness, especially current or previous lung cancer diagnosis or treatment, or ongoing cancer surveillance.

Surgical Resection

Surgical resection was performed when possible via video-assisted thoracoscopic surgery (VATS) lobectomy (Walker, 1999) or via postero-lateral thoracotomy (Shields, 2004). For the VATS approach the surgeon utilised an endoscopic hilar dissection

technique under single-lung anaesthesia. An incision (approximately 5cm in length) was created in the lateral sub-mammary area as a utility port for the passage of large instruments and staplers. This allowed prompt lung collapse and the identification of the most appropriate location for a posterior videothoracoscope port. This provides an excellent view of the thoracic cavity and major fissure similar to the aspect presented to the surgeon at a conventional thoracotomy. A single inferior port was also created which was used at the end of the procedure for the chest drain. Conversion to open thoracotomy was effected by linking the posterior videothoracoscope port and anterior sub-mammary incision which created a virtually identical thoracotomy to that utilised in the standard open technique.

Postero-lateral thoracotomy involved a larger incision with division of the latissimus dorsi muscle and posterior elements of the serratus muscle preserving serratus anterior.

Post-Operative Analgesia

The standard protocol for the unit was intra-operative insertion of a paravertebral catheter for continuous post-operative local anaesthetic administration and intravenous opiate via patient-controlled analgesia (PCA) for the first 24 to 48 hours. Thereafter, patients were converted to oral paracetamol, dihydrocodeine and/or tramadol and/or nefopam.

Clinical Details

Patient demographics, co-morbidities, risk factors, results of investigations and details of surgery and clinical course were obtained from the NHS (National Health Service) confidential medical record and/or direct patient history. These data were obtained with explicit consent and stored electronically without patient identifiable information.

Outcomes

Post-operative trends in biomarkers were analysed for all patients. Patients were then grouped into those with clinical pneumonia and those without clinical pneumonia for analysis. Comparative analysis was also performed of patients undergoing lung resection via VATS or postero-lateral thoracotomy.

Clinical Definitions

Lower respiratory tract infection (LRTI) was defined as new alveolar infiltrates on the chest x-ray (CXR) at 72hrs plus two from three of the following: -

1. Temperature >38.0°C or <35.5°C
2. White blood cell count (WBC) >11.0 or <4.0x10^9/l (on NHS Full Blood Count Analysis)
3. Positive sputum culture.

The CXR at 72hrs was formally reported by a Consultant Radiologist specialising in chest radiology.

Acute Lung Injury (ALI) and Acute Respiratory Distress Syndrome (ARDS) were defined according to the universally applied American-European consensus (Bernard et al., 1994) which is described as: -

1. Acute onset
2. Bilateral infiltrates on CXR
3. PaO_2/FiO_2 ratio <300mmHg for ALI and <200mmHg for ARDS
4. No clinical evidence of left atrial hypertension (or pulmonary artery wedge pressure < 18mmHg if measured).

Summary of Samples Obtained

Samples were obtained from patients pre-operatively and at various time points post-operatively to compare baseline parameters and elucidate temporal changes following surgery. Patients were requested to have venepuncture (30mls of blood), breath tests for measurement of exhaled nitric oxide, bronchoalveolar lavage (BAL) and an infection screen consisting of a CXR, blood culture (20mls of blood), sputum culture, mid-stream specimen of urine (MSSU) and wound swabs at the time points detailed in the table below:

	Pre-Mediastinoscopy	Before Resection	Immediately After Resection	6hrs After Resection	24hrs After Resection	48hrs After Resection	72hrs After Resection
Blood		X		X	X	X	
Breath		X		X	X	X	
BAL	X		X				
Infection Screen							X

Table 2.1 Overview of the Timing of Patient Sampling.

The blood sample at 24hrs also involved arterial blood gas analysis which was usually taken from the arterial pressure monitoring line, or otherwise by radial artery puncture.

Obtaining BAL fluid before surgery and repeating the procedure on the same patient after surgical trauma has not been performed before and provides an ideal opportunity to evaluate the lungs' inflammatory response to surgical trauma.

Blood Preparation

Blood was prepared to separate serum from erythrocytes and enable isolation of granulocytes and mononuclear cells using dextran sedimentation and percoll gradient extraction (Haslett et al., 1985). The blood was sampled in the hospital ward and immediately transferred into a 50ml Becton Dickinson (BD) Falcon tube containing 3mls of Sodium Citrate and transported to the research laboratory. This was adjacent to the hospital at The Queen's Medical Research Institute. It was immediately centrifuged at 350g for 20 minutes with acceleration of 5, brake 0 and temperature 20°C.

All blood preparation was undertaken in a laminar flow hood. The platelet rich plasma (PRP) layer was removed from the centrifuged blood using a plastic pipette, with care taken to avoid disturbing the layers beneath, and transferred into 10ml glass vial tubes, adding 220µl of Calcium Chloride per 10mls. This was left for 1 hour in a water bath at 37°C. The platelet plug was then removed by passage through a BD microfilter leaving the serum. 1ml aliquots of serum were placed into 1.5ml Eppendorf tubes and frozen at -80°C for later analysis. Typically 10 aliquots were frozen at each time point with 1ml of serum retained for use in the same-day experiments.

While the PRP was in the water bath, 6mls of 6% Dextran was added to the erythrocyte layer and topped up to the 50ml mark with 0.9% Sodium Chloride. The tube was inverted a few times to mix. Air bubbles were removed using a plastic pipette. The tube was left to stand for a maximum of 20 minutes or until sedimentation had taken place.

Whilst awaiting sedimentation, Percoll gradients were prepared. 3mls of 10x phosphate buffered saline (PBS) was added to 27mls of Percoll to make up Percoll Stock. From the Percoll Stock, three gradients were prepared in 15ml BD Falcon tubes as follows: -

81% - 8.1mls of Percoll stock plus 1.9mls PBS without calcium and magnesium
68% - 6.8mls of Percoll stock plus 3.2mls PBS without calcium and magnesium

55% - 5.5mls of Percoll stock plus 4.5mls PBS without calcium and magnesium.

The gradients were layered carefully into a 15ml BD Falcon Tube. 3mls of the 81% gradient was layered first followed by 3mls of the 68% solution.

Once sedimentation had taken place, the top layer was carefully removed and transferred to another 50ml BD Falcon tube. This was then topped up to 50mls using 0.9% Sodium Chloride and centrifuged at 350g for 6 minutes, acceleration 9, brake 9 and temperature 20°C. The erythrocytes were discarded. The top layer was discarded and the cell pellet re-suspended in 3mls of the 55% solution. This was carefully pipetted onto the other layered gradients. The layered gradients tube was then centrifuged at 700g for 20 minutes, acceleration 0, brake 0 and temperature 20°C.

Excess fluid was subsequently removed from above the mononuclear layer using a plastic pastette. The mononuclear layer could then be carefully transferred into another 50ml BD Falcon tube. Excess fluid was next removed from above the granulocyte layer allowing the granulocyte layer to be transferred into a separate 50ml BD Falcon tube. Each tube was then topped up to 50mls with PBS and centrifuged at 350g for 6 minutes, acceleration 5 brake 5 and temperature 4°C. The excess fluid was removed from the pellet in each tube and then re-suspended in PBS to the 30ml mark. A NucleoCount® was then undertaken (Chemometec, Denmark). For this, 50µl of cells were mixed with 50µl of Reagent A and 50µl of Reagent B. This mixture was aspirated into the chamber of the NucleoCassette and placed in the NucleoCounter and the cell concentrations recorded.

As a quality control measure, cytospin preparations were performed using 90µl of cell suspensions at a concentration of $1x10^6$/ml plus 10µl serum and the slide spun at 3500rpm for 5 minutes. The slide was then air dried and a Giemsa stain performed before viewing under light microscopy. In addition, 300µl of $1x10^6$/ml cell solution was added to flow cytometry tubes plus 300µl of FACSLyse (BD, UK) for later flow

cytometry analysis (please refer to flow cytometry section for further details) (Figure 2.1).

The mononuclear and granulocyte tubes were then topped up to 50mls with PBS and spun again at 350g for 6 minutes at acceleration 5, brake 5 and temperature 4°C. The cells were finally re-suspended at the desired concentrations in Iscove's Modified Dulbecco's Medium (IMDM) and kept on ice prior to use within a few hours.

Suspended Neutrophil Phagocytosis

Patient neutrophil phagocytosis was evaluated by flow cytometry analysis of neutrophils exposed to fluorescently labelled *Escherichia coli* and Zymosan A (from *Saccharomyces cerevisiae*). Patient neutrophils (prepared as per the blood preparation section) were re-suspended at $10x10^6$/ml in IMDM. 4µl of *Escherichia coli* (K-12 strain) Bioparticles® pre-conjugated with the fluorophore Alexa Fluor® 488 (Invitrogen, UK) was added to an equal volume of autologous patient serum and placed in an incubator at 37°C, 5% CO_2 for 30 minutes to enable opsonisation. 11µl of Zymosan A (from *Saccharomyces cerevisiae*) Bioparticles® pre-conjugated with Alexa Fluor® 594 (Invitrogen, UK) was also mixed with 11µl serum and placed in the incubator for 30 minutes. At the first time point 7 conditions were prepared: -

1. *E. coli* and serum
2. Zymosan A and serum
3. An unstained tube
4. *E. coli* and no serum (negative control)
5. *E. coli* and ice cold serum (negative control)
6. Zymosan A and no-serum (negative control)
7. Zymosan A and ice cold serum (negative control).

At subsequent time points only the fluorophore and serum plus fluorophore and no serum were run. A total volume of 1ml of IMDM was added to each tube with 10% patient serum as indicated. Serum and medium were kept on ice for the cold incubation. $5x10^5$ neutrophils (50µl) were added to each tube. 20µl Zymosan A/serum mix was added to the appropriate tube (cold and warm) and 10µl to the no serum tube. Similarly for *E. coli*, 2µl *E. coli*/serum mix was added to the serum tube and 1µl to the no serum added tube. The tubes were then incubated at 37°C or at 4°C on ice as appropriate for 30 minutes. The fluorophore concentration and duration of experiment was decided upon after a dose and time course trial experiment.

Following incubation, 100µl of 0.4% Trypan Blue was added to each tube to dull any surface bound but not phagocytosed fluorescence probe. Each eppendorf tube was then topped up with PBS and centrifuged at 350g for 4 minutes at 4°C. The supernatant was removed by careful pipetting and the pellet was washed twice more with PBS. The pellet was then resuspended in 300µl FACS™ Lyse solution, transferred to flow cytometry tubes and stored in foil in the cold room until flow cytometry analysis (within 7 days).

A template was created on the flow cytometer (BD Special Order LSR Fortessa) to enable consistent collection of data. The granulocytes were gated on according to size and granularity (Forward Scatter (FSC) and Side Scatter (SSC) respectively). Fluorescence was then measured on the gated cells using channels B 530/30 and YG 610/20 for *E. coli* and Zymosan respectively. The data were subsequently analysed using FlowJo Software (Version 7.6.3) (TreeStar Inc, USA) with the unstained sample used as the basis of the gating for positive phagocytosis.

Plated Neutrophil Phagocytosis

Light microscopy of neutrophil phagocytosis of Zymosan (from *Saccharomyces cerevisiae*) was used as a second method of assessing neutrophil phagocytosis. 250µl

Zymosan (2mg/ml) was added to an equal volume of autologous serum. On a 24-well Costar® plate (Corning, Amsterdam), six wells were used consisting of the following in duplicate; neutrophils alone, neutrophils/zymosan with no serum and neutrophils/zymosan/serum. 1ml IMDM was added with 10% serum as indicated. $5x10^5$ neutrophils (50µl) were added to each well. The plate and zymosan/serum mix were incubated at 37°C 5% CO2 for 30 minutes. Cell adherence to the plate was then confirmed with light microscopy. 10µl Zymosan/serum mix or 5µl Zymosan alone was subsequently added to the appropriate wells and the plate replaced in the incubator for 30 minutes. The culture supernatant was then aspirated from each well and the wells gently washed twice with IMDM. After air drying, Giemsa staining was performed. Positive phagocytosis was defined as the proportion of neutrophils that had ingested two or more zymosan particles as determined by light microscopy. A minimum of 200 cells were counted in each well at two separate positions and the average of the two wells was calculated.

Monocyte Subset Staining For Flow Cytometry

There are at least three subsets of monocytes in humans; classical $CD14^{++}CD16^{-}CCR2^{+}$, non-classical or inflammatory $CD14^{+}$, $CD16^{++}$, CX_3CR1^{+}, and intermediate $CD14^{++}$, $CD16^{+}$. All monocytes express variable levels of the Major Histocompatibility Complex Class II (MHC II) molecule HLA-DR. Evaluation of monocyte subset changes, if any, following thoracic surgery may further help understanding of the inflammatory response.

Following blood preparation (described under Blood Preparation) the mononuclear layer was re-suspended at $2x10^6$/ml in IMDM. Seven conditions were prepared in BD glass flow cytometry tubes; Unstained, CD14, CD16, CCR2, CX_3CR1, HLA-DR and 'Mix'.

0.5mls of the mononuclear layer (i.e. $1x10^6$ cells) was added to each tube plus 50µl mouse serum (Biosera, UK) and left on ice in the cold room for 30 minutes to prevent non-specific binding. Following incubation, the individual fluorescence conjugated antibodies were added to the corresponding labelled tubes in the following concentrations and left for 30 minutes on ice; 1µl (0.2µg) PerCP-Cy™5.5 conjugated mouse IgG_{2a} κ anti-human CD14 (BD, UK), 0.5µl PE-Cy™7 conjugated mouse IgG1 κ anti-human CD16 (BD, UK), 1µl (0.05µg) V450 conjugated mouse IgG_{2a} anti-human HLA-DR (BD, UK), 5µl Alexa Fluor® 647 conjugated mouse IgG_{2b} anti-Human CD192 (CCR2) (BD, UK) and 5µl (0.05µg) FITC conjugated rat $IgG_{2b\,\kappa}$ anti-Human CX_3CR1 (MBL, International Corporation, Mass., USA). All five antibodies were added to the 'Mix' tube. These concentrations were determined following a titration experiment to determine the minimum concentration required to achieve saturating staining based on the manufacturers' recommended volume for flow cytometry as the maximum.

Following incubation, each tube was washed twice with 1ml PBS and centrifuged at 350g for 5 minutes, brake 5, acceleration 5, temperature 4°C. After the final wash, the cells were re-suspended in 400µl of FACSLyse. The cells were later analysed with digital flow cytometry (BD Special Order LSR Fortessa). All seven tubes were used for the pre-resection time point for each patient however at subsequent time points only an unstained and mixed tube were run.

A template was created for 5-colour analysis on the digital flow cytometer. Monocytes were gated on using FSC and SSC properties and each single stain was monitored for fluorescence in the appropriate channel.

Antibody	Fluorescence Channel
HLA-DR	V450
CD16	YG 780/60
CX_3CR1	B530/30
CCR2	R 670/30
CD14	B695/40

Table 2.2. Fluorescence Conjugated Antibodies with Respective Fluorescence Channel on the Flow Cytometer for Monocyte Subset Analysis.

Fluorescent 'spill-over' into other channels was manually compensated digitally for each antibody and the compensation settings applied to all tubes. Subsequent analysis of the compensated data was performed using FlowJo Software (Version 7.6.3) (TreeStar Inc, USA). Monocytes were gated on using FSC and SSC properties and those with positive HLA-DR staining were analysed for CD14 and CD16 staining. CX_3CR1 and CCR2 staining was used to confirm correct identification of specific sub-populations of monocytes.

Stimulation of Monocytes with Lipopolysaccharide (LPS)

The remaining mononuclear cells were centrifuged at 350g, brake 5, acceleration 5 and temperature 4°C for 5 minutes prior to re-suspending in 80µl of buffer (PBS, pH 7.2, 0.5% bovine serum albumin (BSA). 20µl MACS™ CD14 human microbeads (Miltenyi Biotec, Germany) were added and the mixture vortexed and left on ice for 15 minutes. Unbound beads were removed by washing once with 1ml buffer and centrifuging at 350g, acceleration 5, brake 5 temperature 4°C for 10 minutes. The supernatant was then carefully aspirated and the cell pellet re-suspended in 500µl of buffer.

The $CD14^+$ cells (i.e. monocytes) were then separated from $CD14^-$ cells by passage through a magnetic column. The magnet (MiniMACS™ Separator, Miltenyi Biotec,

Germany) was placed on a metal stand and the separating column (MS Column, Miltenyi Biotec, Germany) placed in the designated groove in the middle of the magnet. The column was prepared by adding 500µl of buffer. Once drained, the cells were added into the column and allowed to slowly drain, collecting the $CD14^-$ cells at the bottom in a 15ml BD Falcon tube. The column was then washed three times using 500µl of buffer, collecting the contents into the $CD14^-$ tube. The bound $CD14^+$ cells were then released from the column by removing the magnetic field, adding 1ml of buffer and immediately plunging the contents into a labelled $CD14^+$ 15ml BD Falcon Tube. 4mls of buffer was then added to the $CD14^+$ tube and a cell count performed using the Nucleocounter®. Typically 2-5x10^6 monocytes were isolated and the cells re-suspended in IMDM at 1x10^6/ml.

Monocyte purity was assessed using light microscopy of a cytospin preparation and flow cytometry. For the cytospin, 90µl of cells was added to 10µl of patient serum and the slide spun at 350rpm for 5 minutes. The slide was then air dried and a Giemsa stain performed before viewing under light microscopy.

On a 24-well Costar® (Corning, Amsterdam) flat bottomed cell culture plate, 2 wells were labelled as monocytes + 0 (pre-operatively only) and 2 wells as monocytes + LPS. 500µl IMDM, 50µl serum and 100µl of monocytes (100,000 cells) were added to each well. 100ng of LPS (final concentration 154 ng/ml) from *Escherichia coli* 0127:B8 (Sigma, UK) was added to the two corresponding wells and incubated at 37°C 5% CO_2 for 18 hours. A time course experiment was used to determine this incubation period. The supernatant was then aspirated from each well and placed into 1.5ml labelled eppendorf tubes. These tubes were then centrifuged at 350g for 4 minutes at 4°C in a bench top eppendorf centrifuge. The supernatant was again removed and aliquots frozen at -80°C in eppendorf tubes together with the plates for later analysis.

Bronchoalveolar Lavage

Bronchoalveolar lavage (BAL) was performed under general anaesthesia at two time points using a flexible bronchoscope using a standardised technique as detailed below. Two BALS were performed in each patient studied unless the patient underwent a pneumonectomy where a post-operative BAL was avoided to ensure patient safety. The first was immediately after induction of anaesthesia at the time of the staging bronchoscopy and mediastinoscopy (ie before surgical trauma) (please refer to table 2.1). The second was after completion of lung resection prior to the patient being extubated in theatre (ie after surgical trauma). This required the flexible bronchoscope to be passed through a single lumen endobronchial tube and double lumen tube after the first and second time points respectively.

The contralateral lung to the culprit lesion was lavaged on both occasions with a different lobe lavaged each time. The bronchoscopy and lavage was performed by the Consultant Cardiothoracic Surgeon, Specialist Registrar/Associate Specialist or Research Fellow using a standardised protocol. Once the bronchoscope was wedged in a segmental bronchus, 20mls of 0.9% NaCl was injected through the bronchoscope using a 50ml BD luer lock syringe (representing a bronchiolar sample) and immediately suctioned and discarded. Then 50mls of 0.9% NaCl was injected and the suctioned contents collected in a lavage trap. A further three aliquots of 50mls were injected and again collected in lavage traps. The lavage trap was only changed once nearly full. The lavage contents were pooled into 50ml BD Falcon tubes and transported immediately to the research laboratory on ice for processing.

In a laminar flow hood, the BAL fluid was filtered using BD cell strainers (BD, UK). The strainer was changed when occluded by mucus. The total volume filtered was recorded. Two 100µl aliquots of filtered BAL were placed into 1.5ml eppendorf tubes for cell count enabling the remaining BAL to be centrifuged at 350g, acceleration 5, brake 5 for 10 minutes at 4°C. Meanwhile, the cell count was performed by taking 50µl

of BAL solution and mixing with 50µl of reagent A and B and placing in the Nuclocounter® as previously described (please refer to Blood Preparation Section).

Following centrifugation, the BAL fluid supernatant was carefully poured into a 50ml BD Falcon tube. Ten 1ml aliquots were placed into 1.5ml eppendorf tubes and stored in the freezer at -80°C for later analysis. The cellular pellet was re-suspended in PBS at a concentration of $1x10^6$ cells/ml. Two cytospins were then performed as previously described with 10% serum (please refer to blood preparation section). Once the slides were air dryed, a Giemsa stain was performed. Under light microscopy a differential cell count was performed counting at least 200 cells with two different microscopy fields.

Serum Cytokine Analysis

Serum cytokines were analysed using a commercially available Cytometric Bead Array (CBA) kit (BD Human Inflammatory Cytokines Kit, UK). This kit uses six bead populations with distinct fluorescent intensities coated with capture antibodies specific for IL-8, IL-1β, IL-6, IL-10, TNF-α and IL-12 cytokines. The beads were mixed together with the samples/standards and the PE-conjugated detection antibodies to form sandwich complexes. Data were acquired and quantified using the red channel of a flow cytometer.

Each sample was run in duplicate and a mean calculated for the final patient result. The kits were stored at 4°C in the cold room until use. Reagents were allowed to warm to room temperature for 30 minutes before use.

An aliquot of each serum sample was removed from the -80°C freezer and allowed to thaw thoroughly before use. The samples were run neat. 5µl of each human inflammatory cytokine capture bead (A1-A6) was used per patient sample allowing for an additional 9 standard dilutions. The capture beads (A1-A6) were vortexed before

pipetting the volume required into a 15ml BD Falcon tube. The bead mixture was then vortexed briefly before centrifuging at 200g for 5 minutes at acceleration 5, brake 5, temperature 20°C. The supernatant was carefully aspirated and discarded before re-suspending the pellet in Serum Enhancement Buffer in a volume equal to the volume of supernatant removed. This was then vortexed thoroughly and incubated in the dark for 30 minutes.

One vial of lyophilized standards was transferred into a 15ml BD Falcon tube and re-constituted with 2ml of assay diluent (the 'top' standard). This was allowed to equilibrate for at least 15 minutes before making dilutions. It was mixed carefully with a pipette alone to avoid frothing. A further eight 12x75mm BD Falcon glass tubes were labelled as 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256 and 300µl of assay diluent added to each. Serial dilution was undertaken by transferring 300µl of the top standard to the 1:2 tube and mixed thoroughly by pipette. Further serial dilutions were conducted by transferring 300µl from the 1:2 tube to the 1:4 and so on.

The assay itself was performed in multiple Costar® 96-well plates (Corning, Amsterdam). 25µl of the mixed capture beads was added to each well. 25µl of each standard was added to the first 10 horizontal wells commencing with the assay diluent alone working up to the top standard in well 10. 25µl of each sample was then added to the remaining wells in duplicate. The tubes were incubated for 1.5 hours at room temperature in the dark. After the pipetting for one plate was complete and incubating, other plates could be commenced, running a maximum of 5 plates per day.

1ml of wash buffer was added to each assay tube and centrifuged at 200g for 5 minutes, acceleration 5, brake 5 and temperature 20°C. The supernatant was carefully aspirated and discarded leaving approximately 50µl of solution in each well. 25µl of the PE detection reagent was added and the plate gently agitated to mix. The plate was then incubated in the dark at room temperature for a further 1.5 hours.

Approximately, 15 minutes prior to completion of incubation, the plate reading software was set-up (BD FACSArray). This machine was equipped with a 488 nm laser capable of detecting and distinguishing fluorescence emissions at 576 and 670 nm. Set-up included entering details of the BD kit, number of standards/replicates and plates.

After incubation, 175µl of wash buffer was added to each well using a multi-channel pipette and centrifuged at 200g for 5 minutes, acceleration 5, brake 5 and temperature 20°C. The contents were then discarded before re-suspending the bead pellet in 125µl of wash buffer and analyzing the plates within 3 hours. After the plate reading the fluorescence of the known standards was used to generate a standard curve for each cytokine and convert the fluorescence of the patient samples into an individual cytokine measurement in pg/ml using BD CellQuest™ Software.

Other Cytokine Analysis

The BAL fluid supernatant and LPS-stimulated monocyte supernatant cytokine analysis was also conducted using the Cytometric Bead Array (CBA) Human Inflammatory Cytokines Kit (BD) with important modifications as per the manufacturer's instructions. The capture beads could be simply mixed together without the need for centrifugation and re-suspension in serum enhancement buffer. Further, when pipetting onto the Costar® (Corning, Amsterdam) 96-well plates, the PE detection reagent was added immediately after the capture beads followed immediately by the patient samples. The plate was then incubated in the dark and room temperature for 3 hours before washing and re-suspending in 125µl wash buffer for analysis. BAL fluid samples were run neat in duplicate. The monocyte supernatant cultures were run neat and at 1:50 dilution after a trial dilution series.

Bradford Assay

The total protein content of each well of the monocytes stimulated with LPS was calculated using the Bradford Assay to enable total protein correction of cytokine results. This is a colorimetric protein assay based on the absorbance shift of the dye Coomassie Brilliant Blue G-250. Under acidic conditions, the red form of the dye is converted into its blue form allowing binding to the protein being assayed. Protein binding stabilises the blue form thus the amount of blue dye present is a proportional measure of the total protein, estimated using an absorbance reading.

The plates were thawed to room temperature for approximately 30 minutes. 0.5mls of NaOH was added to each well and a pipette tip used to scratch the surface of each well to ensure the cell layer was mixed. The plate was then allowed to stand for 10 minutes before beginning the assay.

A set of 6 standards was used. A stock solution was prepared of 100mg/ml by adding 1g Bovine Serum Albumin (BSA) to 10mls NaOH. From this stock, a working stock of 100µg/ml was prepared by adding 15µl of 100mg/ml BSA to 15mls NaOH. The standards were then prepared as below:

Standard Concentration	Volume NaOH	Volume 100µg/ml BSA
0µg/ml	10mls	0mls
5µg/ml	9.5mls	0.5mls
10µg/ml	9mls	1ml
20µg/ml	8mls	2mls
30µg/ml	7mls	3mls
40µg/ml	6mls	4mls

Table 2.3. Preparation of Standard Concentrations for Bradford Assay.

In a Costar® 96-well plate (Corning, Amsterdam), 100µl of standards was added to the initial wells in duplicate. 100µl of sample was then added to each well with a careful record kept of the sample ID. This was followed by 100µl of Bradford Reagent which changed colour from brown to blue immediately. The results were then read on a plate reader at 595 nm. The absorbance of the standards vs. their concentration was used to generate a standard curve enabling calculation of the concentration of the unknown samples.

BCA Protein Assay Kit

The above kit was used for the colorimetric detection and quantification of total protein in BAL fluid supernatant (Thermo Scientific, UK). It is based on the reduction of Cu^{+2} to Cu^{+1} by protein in an alkaline medium and the colorimetric detection of Cu^{+1} using a unique reagent containing bicinchoninic acid (BCA). The purple coloured reaction is formed by the chelation of 2 molecules of BCA with one cuprous ion and the absorbance at 562nm is almost linear with increased protein levels over the range 20-2000µg/ml. A series of dilutions of known concentration are prepared from BSA enabling the generation of a standard curve. This enabled calculation of the sample protein concentration.

The kit was removed from cold storage and a set of protein standards were prepared from one Albumin Standard (BSA) ampule of 2mg/ml as detailed in the table below.

Vial	Diluent Volume (0.9% NaCl) (µl)	Vol & Source of BSA (µl)	Final BSA Concentration (µg/ml)
A	0	300 of Stock	2,000
B	125	375 of Stock	1,500
C	325	325 of Stock	1,000
D	175	175 of Vial B Dilution	750
E	325	325 of Vial C Dilution	500
F	325	325 of Vial E Dilution	250
G	325	325 of Vial F Dilution	125
H	400	100 of Vial G Dilution	25
I	400	0	0=Blank

Table 2.4. Preparation of Standard Concentrations for BCA Protein Assay Kit.

The total volume of working reagent (WR) was calculated according to the manufacturers' guidance (Total volume of WR= (# standards + # unknowns) x (# replicates) x (200). The WR was prepared by mixing 50 parts of reagent A with 1 part reagent B.

25µl of each standard and patient sample was pipetted onto a Costar® 96-well plate (Corning, Amsterdam) in duplicate. 200µl of the WR was added to each well and mixed thoroughly for 30 seconds on a plate shaker. The plate was covered and incubated at

37°C for 30 minutes. The plate was then cooled and absorbance measured on a plate reader at 562nm.

Exhaled Nitric Oxide

Fractional Exhaled Nitric Oxide (FENO) was measured from patients pre-operatively and at 6, 24 and 48 hrs post-operatively using a chemilumenescent analyser (Niox® Flex, Aerocrine AB; Smidesvägen, Sweden) and a smaller portable electrochemical sensor (Niox® Mino Aerocrine AB; Smidesvägen, Sweden). All recordings were made at the patient's bedside. The chemiluminescent analyser was set at a mouth flow rate of 50mls/s and a pressure of 10cm H_2O in line with current guidance from The European Respiratory Society /American Thoracic Society recommendations for standardisation of measurements (ATS/ERS, 2005). Two measurements were obtained at each time point and the mean recorded. The machine was calibrated every 14 days and serviced after 18 months as per the manufacturer's guidelines. A further single measurement was taken for comparison with the smaller Niox® Mino device which has identical mouth flow rate and pressure settings.

In addition, the Niox® Flex FlexFlow software was used to measure FENO at two further flow rates of 30mls/s and 100mls/s. These data were used to calculate an estimate of alveolar exhaled nitric oxide and conducting airway exhaled nitric oxide using the two compartment model of NO airway dynamics (Pietropaoli et al., 1999).

Statistical Analysis

The data were analysed using the Minitab 16 Software (Minitab Inc, USA) unless otherwise stated. Normality of distribution was checked using the Anderson-Darling test with a p-value <0.05 considered significant for a not normal distribution. Data that were not normally distributed were $\log_{10}$ transformed prior to further analysis. Pre-operative patient characteristics, type of operation, histopathology and complications

were compared between groups using the two proportion t-test or where numbers were small Fisher's Exact t-test. Post-operative changes were compared using the Paired t-test. Groups of patients were compared using the two-sample t-test. A p value <0.05 was considered significant. A repeated measures ANOVA model was created to incorporate variance attributable to time points, type of surgery and pneumonia versus no pneumonia. To determine relative risk for pneumonia, Youden's test (18) was applied to parameters which were significantly higher in patients going on to develop pneumonia. Having established the optimal cut-off for predicting pneumonia, the relative risk for each parameter was determined using Fisher's test. Statistical significance was considered to be at the $p<0.05$ level. The statistical program GraphPad Prism 5 (GraphPad Software Inc. USA) was used for this purpose. A change in innate immune parameters of 10% was considered to be clinically significant. 40 patients were required to have a 90% power to detect a difference with a significance level of 0.01.

List of Reagents/Key Products

Alexa Fluor® 647 conjugated mouse IgG_{2b} anti-Human CD192 (CCR2) (BD, UK)
BD Cytometric Bead Array (CBA) Human Inflammatory Cytokines Kit II
BD FACS Lysing Solution 10x concentrate (BD, UK)
Bovine Serum Albumin (BSA) (Sigma, UK)
Bradford Reagent (Sigma, UK)
Buffer containing Phosphate Buffered Saline (PBS), pH 7.2, 0.5% bovine serum albumin (BSA)
Calcium Chloride
CD14 Human Microbeads (Miltenyi Biotec, Germany)
Escherichia coli (K-12 strain) Bioparticles®, Alexa Fluor® 488 conjugate (Invitrogen, UK)
FITC conjugated rat $IgG_{2b\,\kappa}$ anti-Human CX_3CR1 (MBL, International Corporation, Mass., USA)
Iscove's Modified Dulbecco's Medium (IMDM) (Invitrogen, UK)

Lipopolysaccharide (LPS) from *Escherichia coli* 0127:B8 (Sigma-Aldrich, USA)

MiniMACS™ Separator (Miltenyi Biotec, Germany)

Mouse Serum (Biosera, UK)

MS Columns (Miltenyi Biotec, Germany)

Nucleocounter® NC-100™ Reagent A and Reagent B (Chemometec A/S, Denmark)

Niox® Flex, Aerocrine AB; Smidesvägen, Sweden

Niox® Mino Aerocrine AB; Smidesvägen, Sweden

Pecoll (GE Healthcare)

PE-Cy™7 conjugated mouse IgG1 κ anti-human CD16 (BD, UK)

PerCP-Cy™5.5 conjugated mouse IgG_{2a} κ anti-human CD14 (BD, UK)

Phosphate Buffered Saline (PBS) without calcium and magnesium X1 and x10 concentrate (Sigma, UK)

Pierce® BCA Protein Assay Kit (Thermo Scientific®, UK)

Quantichrom™ Urea Assay Kit (BioAssay Systems Ca. USA)

Sodium Citrate

Trypan Blue 0.4% (Sigma, UK)

V450 conjugated mouse IgG_{2a} anti-human HLA-DR (BD, UK)

Zymosan A (*Saccharomyces cerevisiae*) Bioparticles® Alexa Fluor® 594 conjugate (Invitrogen, UK)

Zymosan A (*Saccharomyces cerevisiae)* (Sigma, UK)

0.1M NaOH (2g of NaOH to 500mls of distilled water)

0.9% Sodium Chloride

6% Dextran

III - RESULTS – Innate Immune Function Following Major Anatomic Lung Resection for Clinically Suspected Lung Cancer

This chapter evaluates the post-operative innate immune response to major lung resection for suspected lung cancer. As well as describing general trends, comparisons were drawn between lung resection undertaken utilising VATS and conventional thoracotomy with the hypothesis that VATS would induce less trauma and thus disturbance in immune function.

Patient Background

A total of 40 patients were included in the study. Sixty-five percent (n=26) of lung resections were performed utilising a VATS approach with the remainder undergoing conventional postero-lateral thoracotomy (35%, n=14). The type of surgery performed was determined on clinical grounds. Three out of the 14 thoracotomy patients (20%) were potential VATS cases for whom VATS resection was found technically inappropriate leading to early conversion to thoracotomy and conventional open resection.

The key clinical characteristics of patients in the two surgical groups are detailed in Table 3.1. There were notably more males in the thoracotomy group (78.6% cf. 46.2% p=0.027). Mean blood loss was lower in VATS patients (geometric mean 299 SD 2.3 cf. 88 SD 2.2 $p<0.0005$) although there was no significant difference in the post-operative reduction in blood haemoglobin levels (13.6g/l SD 11.5 for thoracotomy and 12.7g/l SD 9.4 for VATS p=0.785). Mean duration of surgery was shorter for thoracotomy patients (182 minutes SD 36.7 cf. 211 SD 44.8 p=0.034) but length of post-operative stay was longer (geometric mean 8.2 SD 1.9 cf. 5.2 SD 1.4 p=0.024). Although the Thoracoscore was slightly higher for the thoracotomy patients, no statistically significant difference between the two groups was identified (3.1% SD 2.5 for thoracotomy cf. 1.9% SD 1.0 for VATS p=0.103).

The final histopathological diagnosis and TNM stage are shown in Tables 3.2 and 3.3 respectively. While the study targeted primary lung cancer, two patients had a different post-operative diagnosis (one was metastatic colorectal carcinoma and the other benign Wegener's granulomatosis). Not surprisingly, VATS surgery had more patients with early stage IA disease compared to thoracotomy (7.7% cf. 46.2% p=0.002). The specific operation performed for each patient is shown in Table 3.4. In general terms, thoracotomy involved a few higher risk operations including sleeve resection, chest wall resection and pneumonectomy in addition to lobectomies like the VATS group.

Characteristic	All Patients n=40	Thoracotomy n=14	VATS n=26	p-value
Male, n (%)	23 (57.5)	11 (78.6)	12 (46.2)	**0.027**
Mean Age, years (SD)	66.9 (7.8)	66.6 (7.8)	66.7 (8.1)	0.975
Mean BMI, kg/m^2 (SD)	26.6 (4.3)	26 (3.9)	27 (4.5)	0.418
Current Smoker, n (%)*	13 (32.5)	6 (42.3)	7 (26.9)	0.314
Mean FEV_1, % Predicted (SD)	87.3 (23.8)	80.1 (19.3)	93.0 (25.0)	0.078
Mean FEV_1/FVC, % Predicted (SD)	88.5 (13.9)	87.2 (15.6)	88.6 (13.3)	0.778
Mean T_{CO}, % Predicted (SD)	72.0 (21.9)	66.6 (18.4)	74.8 (23.3)	0.231
Mean K_{CO}, % Predicted (SD)	78.1 (17.6)	79.0 (15.8)	80.1 (18.8)	0.849
Mean Thoracoscore, % (SD)	2.5 (1.7)	3.1 (2.5)	1.9 (1.0)	0.103
Geometric Mean Blood Loss (SD)	139 (2.7)	299 (2.3)	88 (2.2)	**<0.0005**
Mean Change in Blood Hb g/l (Pre-op-Day 1), n (SD)	13.8 (10.0)	13.6 (11.5)	12.7 (9.4)	0.785
Mean Duration of Surgery, Minutes (SD)	203 (44.4)	182 (36.7)	211 (44.8)	**0.034**
Geometric Mean Post-Operative Stay (SD)	6.3 (1.7)	8.2 (1.9)	5.2 (1.4)	**0.024**

*Current or ex <2 months duration

Table 3.1 Clinical Characteristics of Patients Undergoing Lung Resection. Data are presented as means (and standard deviation) or n (%) as appropriate. P values refer to the comparison of VATS versus thoracotomy groups, with statistical analysis using an unpaired t-test for continuous variables or the two proportion test for non-continuous variables (or Fisher's exact test where numbers were small). Data that were not normally distributed (as determined by the Anderson-Darling test) were log_{10} transformed prior to further analysis with the data reported as a geometric mean. Prior to transformation blood loss was reported in millilitres (mls) and post-operative stay in days. BMI, body mass index; FEV_1, forced expiratory volume in 1 second; FVC, forced vital capacity; Thoracoscore is risk stratification system for inpatient mortality using a number of pre-operative patient characteristics (Falcoz et al., 1997); Hb, haemoglobin; Tco, transfer factor for carbon monoxide; Kco, transfer factor for carbon monoxide, corrected for lung volume.

Final Histopathology Diagnosis & TNM Lung Cancer Stage	All Patients n=40	Thoracotomy n=14	VATS n=26	p-value
Adenocarcinoma, n (%)	17 (42.5)	1 (7.1)	16 (61.5)	**0.001**
Squamous Cell Carcinoma, n (%)	14 (35.0)	9 (64.2)	5 (19.2)	**0.003**
Bronchoalveolar Carcinoma, n (%)	3 (7.5)	0 (0.0)	3 (11.5)	0.539
Large Cell Carcinoma, n (%)	1 (2.5)	1 (7.1)	0 (0.0)	0.350
Pleomorphic Carcinoma, n (%)	1 (2.5)	0 (0.0)	1 (3.8)	1.000
Synchronous Pleomorphic Carcinoma & Adenocarcinoma, n (%)	1 (2.5)	1 (7.1)	0 (0.0)	0.350
Atypical Carcinoid, n (%)	1 (2.5)	1 (7.1)	0 (0.0)	0.350
Metastatic Colorectal Adenocarcinoma, n (%)	1 (2.5)	0 (0.0)	1 (3.8)	1.000
Wegener's Granulomatosis, n (%)	1 (2.5)	1 (7.1)	0 (0.0)	0.350

Table 3.2 Final Histopathological Diagnosis from Resected Lung Specimens. P-values refer to the comparison between VATS and thoracotomy groups and were calculated using the two proportion test (or Fisher's exact test where numbers were small).

Lung Cancer TNM Stage	All Patients n=38 n (%)	Thoracotomy n=13 n (%)	VATS n=25 n (%)	p-value
Total Stage IA	**13 (34.2)**	**1 (7.7)**	**12 (48.0)**	**0.015**
T1a N0 M0	6 (15.8)	0 (0)	6 (24.0)	
T1b N0 M0	7 (18.4)	1 (7.7)	6 (24.0)	
Total Stage IB	**8 (21.1)**	**1 (7.7)**	**7 (28.0)**	0.222
T2a N0 M0	8 (21.1)	1 (7.7)	7 (28.0)	
Total Stage IIA	**6 (15.8)**	**3 (23.1)**	**3 (12.0)**	0.392
T2b N0 M0	1 (2.6)	0 (0)	1 (4.0)	
T1a N1 M0	1 (2.6)	0	1 (4.0)	
T1b N1 M0	1 (2.6)	1 (7.7)	0 (0)	
T2a N1 M0	3 (7.9)	2 (15.4)	1 (4.0)	
Total Stage IIB	**5 (5.2)**	**2 (15.4)**	**0 (0)**	0.111
T2b N1 M0	1 (2.6)	1 (7.7)	0 (0)	
T3 N0 MX	1 (2.6)	1 (7.7)	0 (0)	
Total Stage IIIA	**8 (21.1)**	**5 (38.5)**	**3 (12.0)**	0.094
T2b N2/N3 M0	1 (2.6)	1 (7.7)	0 (0)	
T3 N1 M0	2 (5.3)	2 (15.4)	0 (0)	
T3 N2 M0	2 (5.3)	2 (15.4)	0 (0)	
T4 N0 M0	3 (7.9)	0 (0)	3 (12.0)	
Total Stage IIIB	**1 (2.6)**	**1 (7.7)**	**0 (0)**	0.342
T4 N2 M0	1 (2.6)	1 (7.7)	0 (0)	

Table 3.3 Final Pathological Lung Cancer TNM Stage. P-values refer to the comparison between VATS and thoracotomy groups were calculated using the Fisher's exact test. Significant p-values are shown in bold ($p<0.05$). Earlier stage tumours were targeted by VATS.

Lung Resection	All Patients n=40 n (%)	Thoracotomy n=14 n (%)	VATS n=26 n (%)	p-value
Left Upper Lobectomy	14 (35.0)	5 (35.7)	9 (34.6)	0.945
Left Lower Lobectomy	1 (2.5)	0 (0.0)	1 (3.8)	1.000
Right Upper Lobectomy	10 (25.0)	0 (0.0)	10 (38.4)	**0.007**
Right Middle Lobectomy	4 (10.0)	1 (7.1)	3 (11.5)	1.000
Right Lower Lobectomy	2 (5.0)	1 (7.1)	1 (3.8)	1.000
Right Middle & Lower Lobectomy	2 (5.0)	1 (7.1)	1 (3.8)	1.000
Right Basal Segmentectomy	1 (2.5)	0 (0.0)	1 (3.8)	1.000
Left Upper Lobectomy with Vascular Sleeve	1 (2.5)	1 (7.1)	0 (0.0)	0.350
Left Upper Lobectomy with Vascular & Bronchial Sleeve	1 (2.5)	1 (7.1)	0 (0.0)	0.350
Left Lower Lobectomy & Wedge Left Upper Lobe	1 (2.5)	1 (7.1)	0 (0.0)	0.350
Right Upper Lobectomy, Wedge Right Middle & Chest Wall Resection	1 (2.5)	1 (7.1)	0 (0.0)	0.350
Left Pneumonectomy	1 (2.5)	1 (7.1)	0 (0.0)	0.350
Right Pneumonectomy	1 (2.5)	1 (7.1)	0 (0.0)	0.350

Table 3.4 Anatomical Lung Resection Performed. All operations were combined with an adenectomy. P-values were calculated using the two-proportion t-test or where numbers were small using the Fisher's exact test. Significant p-values are shown in bold ($p<0.05$).

Post-operative complications are detailed in Table 3.5. Criteria for diagnosis of pneumonia and ALI/ARDS were defined prospectively and are specified in the methodology chapter (Chapter II, Clinical Definitions). Excessive pain was defined as pain not controlled with the usual post-operative analgesia regimen (described in Chapter II, Post-Operative Analgesia) and urinary tract infection as positive culture on a MSSU. Overall, there were no in-patient deaths or ALI/ARDS in both surgical groups. 21/40 (52.5%) patients did not suffer a complication. The most common post-operative complication was chest infection (35%) followed by prolonged air leak (17.5%). There were no statistically significant differences found for complications between the surgical groups.

Post-Operative Complication	All Patients n=40 n (%)	Thoracotomy n=14 n (%)	VATS n=26 n (%)	p-value
In-patient Mortality	0 (0.0)	0 (0.0)	0 (0.0)	1.000
Respiratory Failure requiring Re-intubation	1 (2.5)	1 (7.1)	0 (0.0)	0.350
Mini-Tracheostomy and/or Suction Bronchoscopy	2 (5.0)	1 (7.1)	1 (3.8)	1.000
Pneumonia	14 (35.0)	5 (35.7)	9 (34.6)	0.945
Bleeding requiring Surgical Re-exploration	1 (2.5)	1 (7.1)	0 (0.0)	0.350
Stroke	1 (2.5)	1 (7.1)	0 (0.0)	0.350
Bowel Obstruction	1 (2.5)	0 (0.0)	1 (3.8)	1.000
Acute Renal Failure	1 (2.5)	1 (7.1)	0 (0.0)	0.350
Air Leak >5 days	7 (17.5)	3 (21.4)	4 (15.4)	0.679
Atrial Fibrillation	5 (12.5)	3 (21.4)	2 (7.7)	0.322
Pericarditis	1 (2.5)	1 (7.1)	0 (0.0)	0.350
Temporary Heart Block (2nd Degree)	1 (2.5)	1 (7.1)	0 (0.0)	0.350
Clostridium difficile Diarrhoea	1 (2.5)	0 (0.0)	1 (3.8)	1.000
Acute Urinary Retention	1 (2.5)	0 (0.0)	1 (3.8)	1.000
Urinary Tract Infection	1 (2.5)	0 (0.0)	1 (3.8)	1.000
Wound Cellulitis	1 (2.5)	1 (7.1)	0 (0.0)	0.350
Excessive Pain	3 (7.5)	1 (7.1)	2 (7.7)	1.000
Opiate Toxicity/Confusion/Agitation	3 (7.5)	2 (14.3)	1 (3.8)	0.276
Nausea and Vomiting	1 (2.5)	1 (7.1)	0 (0.0)	0.350
None	21 (52.5)	6 (42.9)	15 (57.7)	0.366

Table 3.5 Post-Operative Complications Following Lung Resection. P values refer to the comparison between VATS and thoracotomy groups, calculated using the two proportion test (or Fisher's exact test where numbers were small). Pneumonia was pre-defined as new alveolar infiltrates on a chest x-ray at 72 hours, reported by an experienced pulmonary radiologist, plus two from three of: temperature >38.0°C or <35.5°C; white blood cell count (WBC) >11.0 or <4.0x10^9/L; and positive microbiology on sputum culture. Excessive pain was defined as pain not controlled with the usual post-operative analgesia regimen.

Serum Cytokines: IL-6, IL-8, IL-10, TNF-α, IL-12 and IL-1β

Cytokines are small cell-signalling protein molecules which modulate immune responses. In order to evaluate general inflammatory trends post-operatively, serum cytokines were measured. Post-operative increases in serum IL-6, IL-8 and IL-10 were found as detailed in Figures 3.1-3.3. For all patients IL-6, IL-8 and IL-10 followed similar kinetics with a rapid increase at 6hrs, peaking at 24hrs before declining at 48hrs. Interestingly IL-6 peaked at 6hrs in thoracotomy patients before falling. All changes were significant compared with the pre-operative value ($p<0.0005$). VATS patients induced lower levels of cytokine at each time point compared to thoracotomy. For IL-6 this was statistically significant at 6hrs using a two-sample t-test (geometric mean ratio 1.61 95% CI 1.06-2.46 $p=0.028$). ANOVA modelling confirmed a significant post-operative trend for lower levels of IL-6 ($p=0.026$), IL-8 ($p=0.018$) and IL-10 ($p=0.047$) in VATS compared to thoracotomy patients.

There were no statistically significant post-operative changes for TNF-α, IL-12 or IL-1β (Figure 3.4). The trend for lower cytokine levels in VATS patients remained which was significant for TNF-α (ANOVA $p=0.031$).

A comparison was made between pre-operative patient serum cytokines and a mixed-gender population of a similar age without active cancer. Table 3.6 confirms the groups were well matched in terms of age, gender and smoking status. Table 3.7 reveals small but statistically significant differences between the groups for IL-6 (geometric mean ratio 1.78 95% CI 1.13-2.79 $p=0.017$), IL-12 (mean difference 1.39pg/ml 95% CI 0.075-2.705 $p=0.039$) and TNF-α (mean difference 1.70pg/ml 95% CI 0.252-3.143 $p=0.023$). No statistically significant differences were found for IL-8, IL-1β and IL-10.

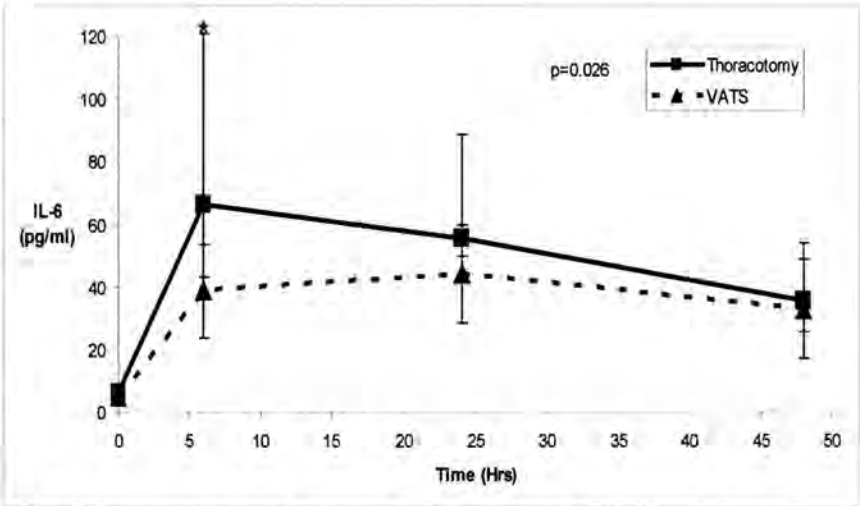


Figure 3.1 Post-Operative Trend in Serum IL-6. Individual trend lines are shown for thoracotomy and VATS groups. The data was not normally distributed thus the median values are shown. Error bars represent the interquartile range. The data was $\log_{10}$ transformed prior to undertaking paired T-tests. There was an overall significant increase in IL-6 from pre-operative value at all time points ($p<0.0005$). VATS also induced a lower change in IL-6 levels compared with thoracotomy which was statistically significant at 6hrs post-operatively on a two sample t-test ($p=0.028$) denoted by the * symbol. ANOVA modelling confirmed significant change post-operatively for all patients ($p<0.0005$) and lower levels of IL-6 for VATS compared to thoracotomy patients ($p=0.026$). n=40 for all time points except n=38 at 48hrs.

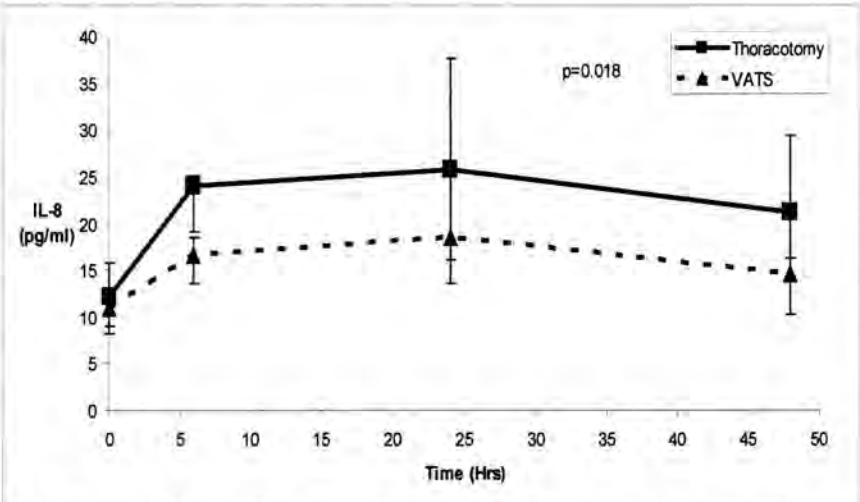


Figure 3.2 Post-Operative Trend in Serum IL-8. Individual trend lines are shown for thoracotomy and VATS groups. The data was not normally distributed thus the median values are shown. Error bars represent the interquartile range. The data was $\log_{10}$ transformed prior to undertaking paired T-tests. There was a significant change from pre-operative value at all time points ($p<0.0005$) with IL-8 peaking at 24hrs ($p<0.0005$). There was no statistically significant difference between VATS and thoracotomy at any specific individual time point. ANOVA modelling confirmed significant change post-operatively for all patients ($p<0.0005$) with VATS surgery inducing lower levels of IL-8 compared to thoracotomy patients ($p=0.018$). n=40 for all time points except n=38 at 48hrs.

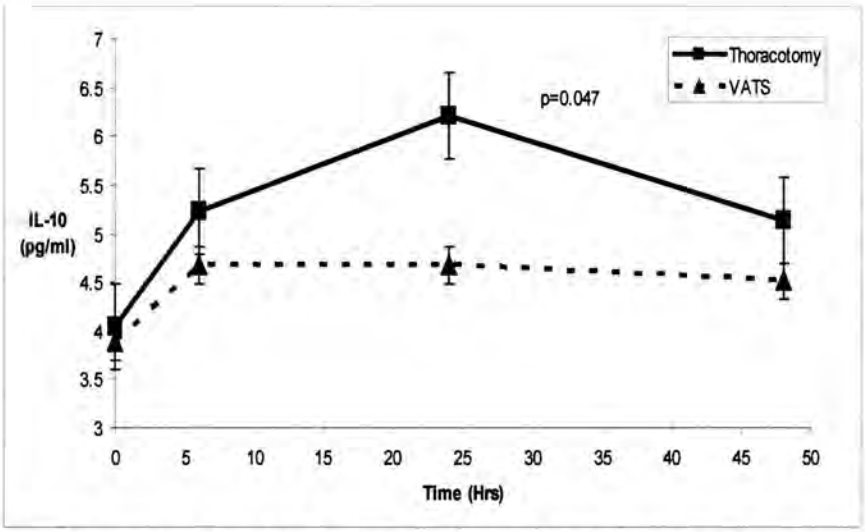


Figure 3.3 Post-Operative Trend in Serum IL-10. Individual trend lines are shown for thoracotomy and VATS groups. The mean values are shown with error bars representing the standard error of the mean. Paired T-tests did not reveal a significant post-operative change at any time point ($p<0.05$). However, ANOVA modelling revealed a significant increase in IL-10 post-operatively ($p<0.0005$) peaking at 24hrs. VATS surgery induced lower levels of IL-10 compared with thoracotomy on ANOVA modelling ($p=0.047$). n=40 for all time points except n=38 at 48hrs.

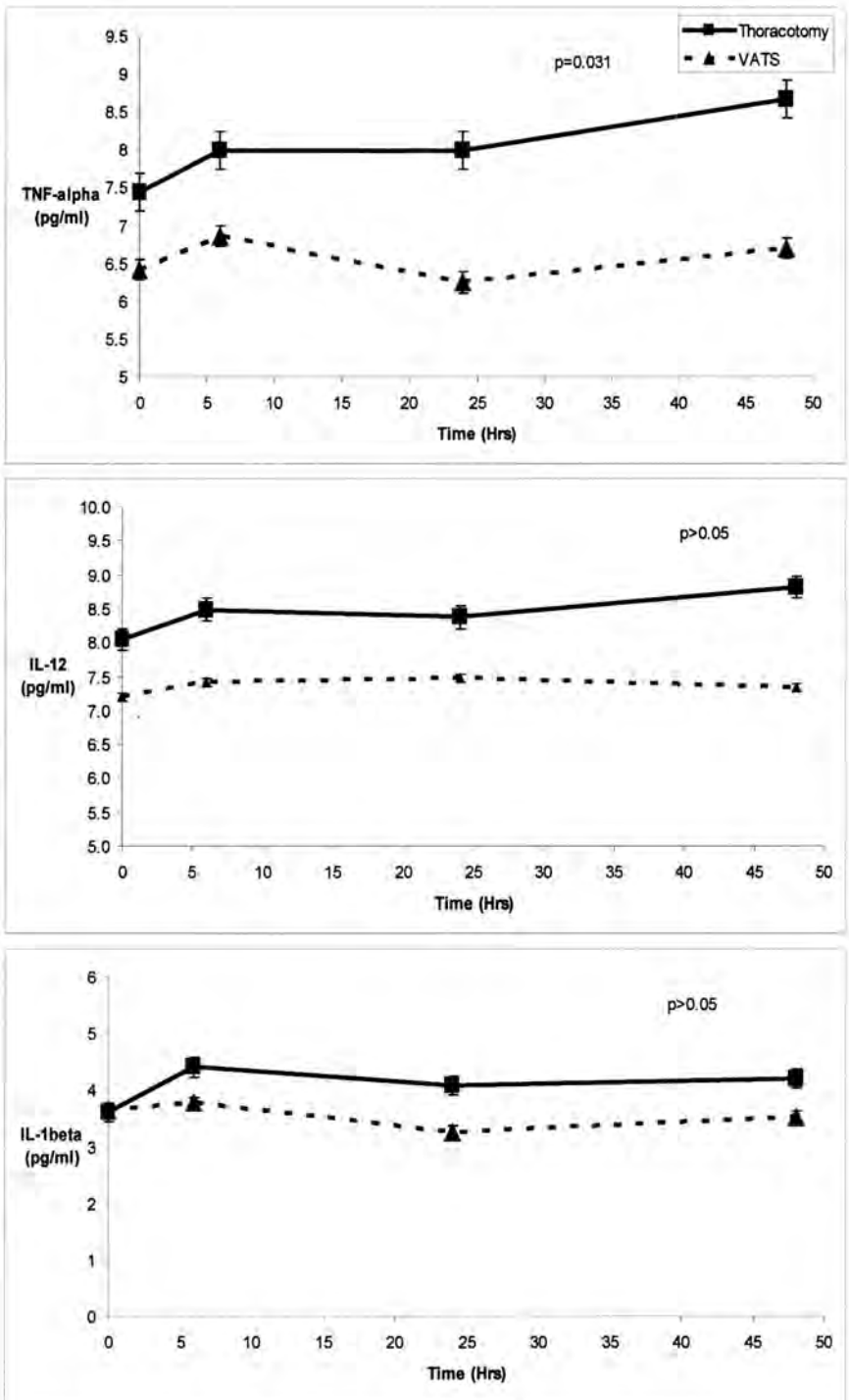


Figure 3.4 Post-Operative Trends in TNF-α, IL-12 and IL-1β. Individual trend lines are shown for thoracotomy and VATS groups. Mean values are shown with error bars representing the standard error. No statistically significant post-operative change was found. No significant difference between VATS and thoracotomy groups was found using two-sample t-tests although ANOVA modelling identified lower levels of TNF-α in VATS patients compared with thoracotomy (ANOVA p=0.031). n=40 for all time points except n=38 at 48hrs.

Characteristic	Control Group n=10	Patients n=40	p-value
Male, n (%)	6 (60.0%)	23 (57.5%)	0.885
Mean Age, Years (SD)	69.1 (5.7)	66.6 (7.9)	0.272
Current Smoker, n (%)	3 (30.0)	13 (32.5)	0.878

Table 3.6 Comparison of the Demographics and Smoking Status of the healthy control group and patients. The two proportion t-test was used to compare the groups and no statistically significant difference was found.

Cytokine	Control Population (pg/ml)	Pre-Operative Patient (pg/ml)	p-value
IL-12	6.10 (1.60)	7.49 (2.39)	**0.039**
TNF-α	5.07 (1.71)	6.77 (2.79)	**0.023**
IL-10	3.60 (1.01)	3.95 (1.09)	0.363
IL-6*	3.30 (2.31-3.54)	5.33 (4.19-6.36)	**0.001**
IL-8*	8.80 (7.19-10.26)	11.35 (8.76-13.43)	0.061
IL-1β	2.78 (1.34)	3.62 (1.13)	0.093

Table 3.7 Healthy Control Population vs. Pre-operative Patient Serum Cytokines. IL-6 and IL-8 data were not normally distributed and were $\log_{10}$ transformed prior to further analysis. A two sample t-test was used to compare the groups. Mean values with standard deviation in brackets are shown except for IL-6 and IL-8 where median values are shown together with interquartile range in brackets (denoted by *). Serum levels of IL-6, IL-12 and TNF-α were found to be slightly higher in lung resection patients pre-operatively than healthy controls of a similar age ($p<0.05$). No significant difference was found for IL-1, IL-8 and IL-10. N=40 for patients and n=10 for controls.

Post-Operative Changes in Blood Leucocyte Count, Platelets and Albumin

In addition to serum cytokine responses as a measure of inflammation, routine peri-operative blood parameters were recorded and analysed. Lung surgery invoked major changes in the leucocyte cell counts (Figure 3.5). There was a post-operative leucocytosis which rapidly increased on day one post-operatively with a small further increase up to day 3 post-operatively (geometric mean ratio 1.42 95% CI 1.32-1.54 $p<0.0005$). This was caused primarily by a neutrophilia (geometric mean ratio 1.83 95% CI 1.66-2.01 $p<0.0005$) and monocytosis (geometric mean ratio 1.37 95% CI 1.22-1.54 $p<0.0005$). Interestingly, there was a reduction in the lymphocyte count for all patients which remained depressed up to three days post-operatively (geometric mean ratio 1.75 95% CI 1.58-1.95 $p<0.0005$). No significant difference was found between VATS and thoracotomy patients. These trends were confirmed on ANOVA modelling.

The platelet count and serum albumin fell post-operatively for all groups of patients (Figures 3.6 and 3.7). Platelet count reached a trough at day 2 (geometric mean 1.14 95% CI 1.09-1.2 $p<0.0005$) before increasing on day 3. Serum albumin fell post-operatively to a trough on day 2 although no measurements beyond day 2 were performed (mean difference 7g/l 95% CI 5.91-9.52 $p<0.0005$). ANOVA modelling noted significantly higher overall levels of serum albumin in VATS patients compared with thoracotomy as detailed in Figure 3.7 ($p=0.001$).

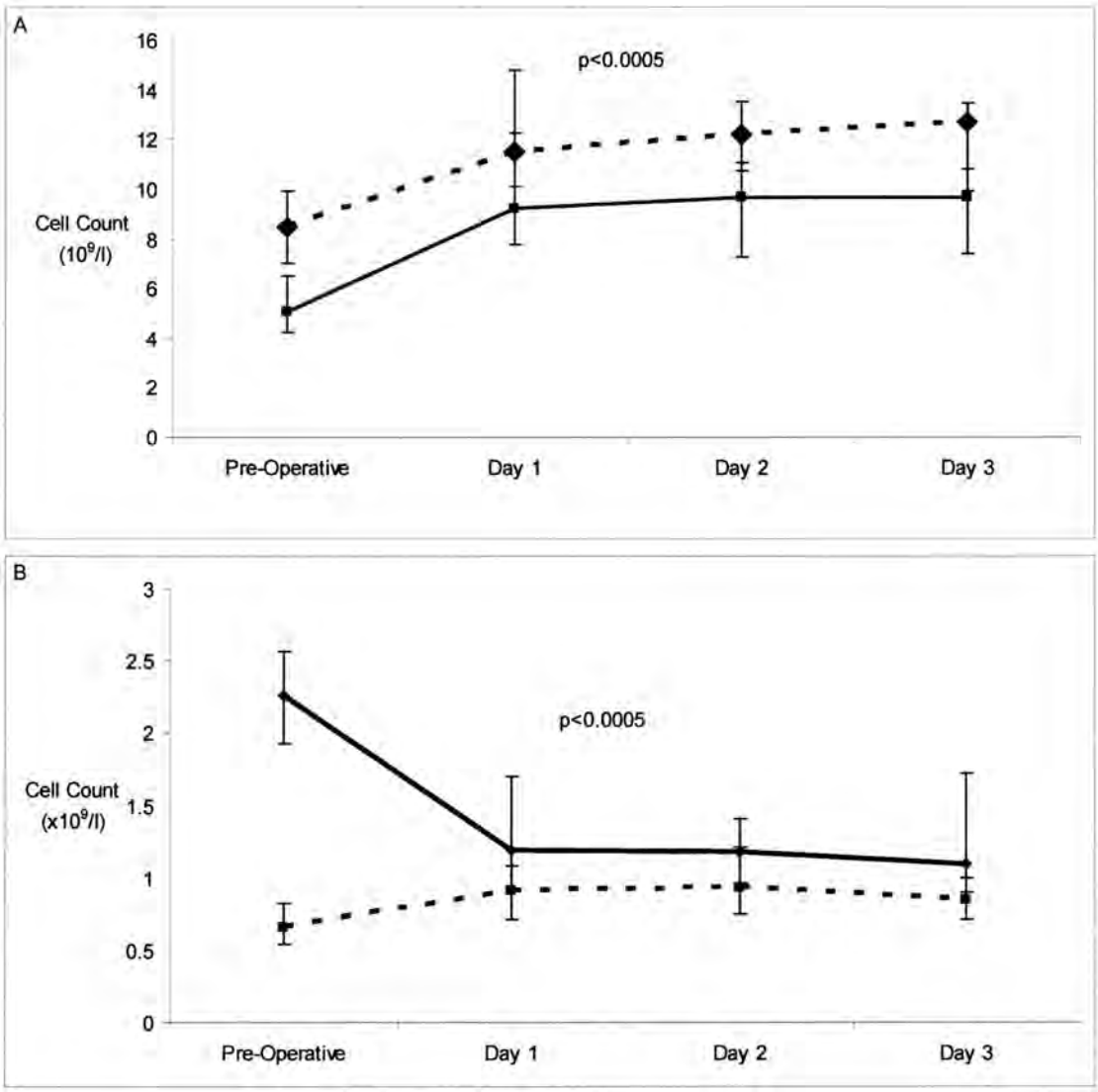


Figure 3.5 Post-Operative Changes in Leucocytes. [A] Serial total leucocyte (broken line) and neutrophil counts (continuous line) in whole blood. Due to not normal distribution, data are presented as medians, error bars represent interquartile range. ANOVA modelling revealed a significant post-operative leucocytosis ($p<0.0005$) and neutrophilia ($p<0.0005$). N=40 for pre-operative and day 1 samples, n=30 on day 2 and n=26 on day 3. [B] Serial lymphocyte (continuous line) and monocyte (broken line) counts in whole blood. Data are presented as medians, error bars represent interquartile range. ANOVA modelling revealed a significant monocytosis ($p<0.0005$) and a relative lymphopenia post-operatively ($p<0.0005$). N=40 for pre-operative and D1 samples, n=30 on day 2 and n=26 on day 3. All data were obtained from the NHS Lothian Haematology laboratory.

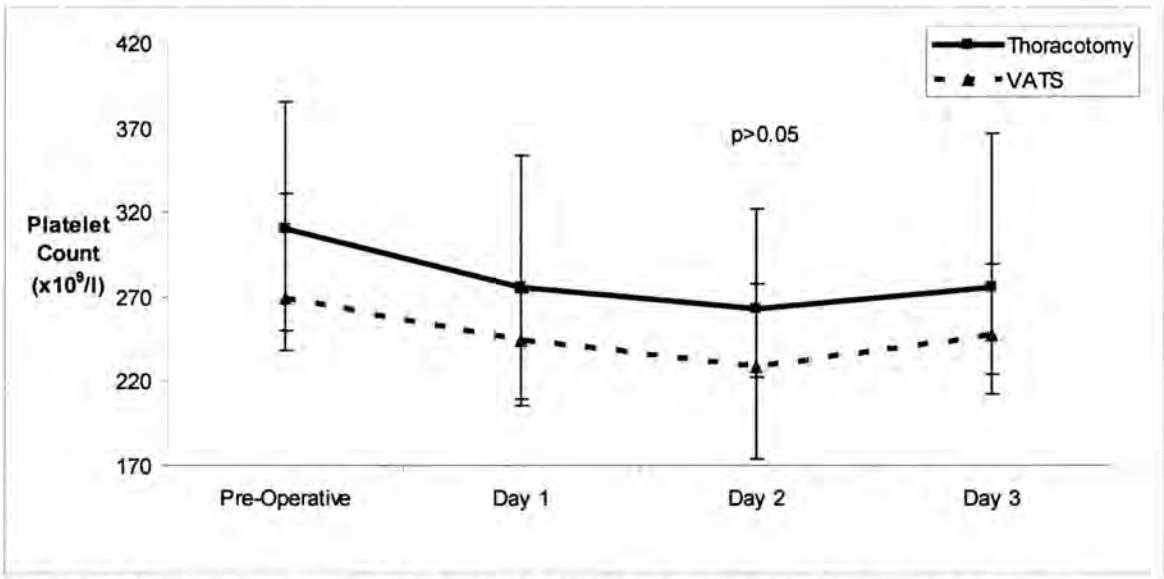


Figure 3.6 Post-operative Trend in Platelet Count. The data points represent the median platelet count at each time point. Error bars represent the interquartile range. The data was $\log_{10}$ transformed due to not normal distribution of data as determined by the Anderson-Darling test. Paired T-tests and ANOVA modelling revealed a significant post-operative reduction in platelet count ($p<0.0005$). Two sample t-tests and ANOVA modelling found no difference in post-operative change between VATS and thoracotomy ($p>0.05$). Note n=40 for pre-operative and day 1 samples but n=30 on day 2 and n=26 on day 3 samples. The data were obtained from the NHS Lothian Haematology Laboratory.

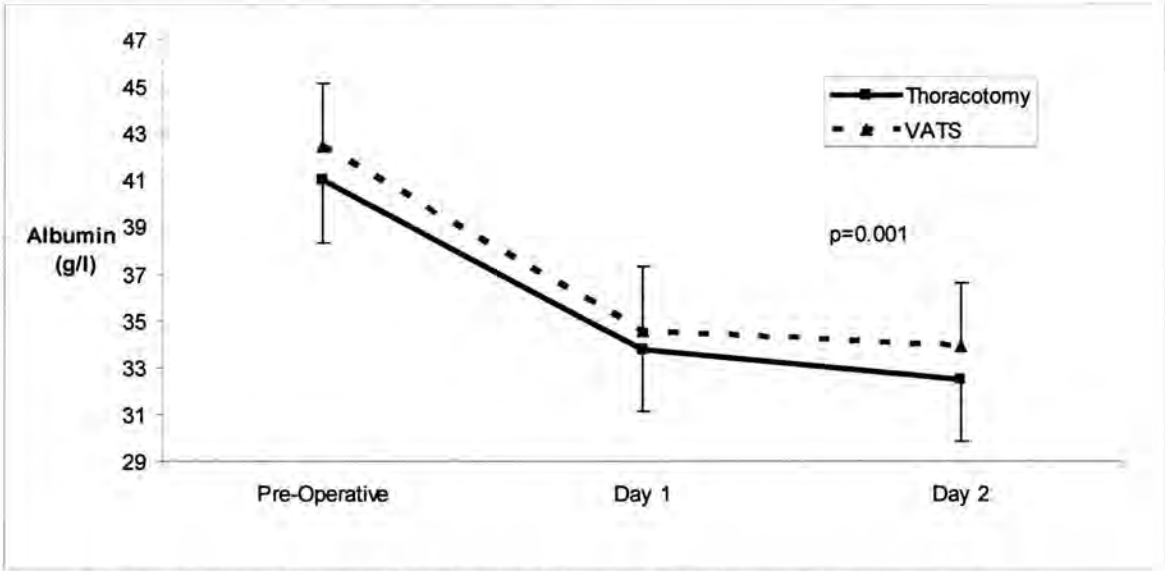


Figure 3.7 Post-Operative Trend in Serum Albumin. The data points represent the mean serum albumin level of all patients. Error bars represent standard errors of the mean. Paired T-tests and ANOVA modelling revealed a significant post-operative reduction in serum albumin ($p<0.0005$). There was a significant difference found between VATS and thoracotomy on ANOVA modelling ($p=0.001$). Note n=40 for pre-operative and n=34 for day 1 samples. n= 21 on day 2. The data were obtained from the NHS Biochemistry laboratory.

Blood Neutrophil Phagocytosis

A neutrophilia is well described after any significant surgical trauma. The function of neutrophils post-lung resection is however less well characterised. Neutrophils were isolated from whole blood using dextran sedimentation and Percoll extraction. Figure 3.8 [A] shows the purity of granulocytes achieved which was confirmed on cytospin with Giemsa staining to be >99% neutrophils. Phagocytosis was then assessed using fluorescently labelled *E.coli* and Zymosan assays which were assessed on flow cytometry as described in Figure 3.9. The plated zymosan phagocytosis assay was assessed by light microscopy as described in Figure 3.10.

A summary of the trends in post-operative neutrophil phagocytosis is shown in Figure 3.11. Suspended neutrophil phagocytosis of Zymosan pre-conjugated with Alexa Fluor® 594 was most variable post-operatively with a significant trend towards reduction on ANOVA modelling ($p=0.047$). There was no significant change in *E. coli* pre-conjugated with Alexa Fluor® 488 ($p=0.318$) or plated Zymosan phagocytosis ($p=0.080$) on ANOVA modelling which remained generally high for all patients.

Paired T-tests did not identify a significant reduction in suspended fluorescently labelled Zymosan phagocytosis post-operatively ($p=0.098$ at 24hrs and 48hrs) but did reveal a significant reduction in plated neutrophil Zymosan phagocytosis at 24hrs ($p=0.018$) and an increase in *E. coli* phagocytosis at 48hrs ($p=0.028$) compared to pre-operative values. No significant difference between VATS and thoracotomy patients were found ($p>0.05$, data not shown). There were also no significant differences in pre-operative phagocytosis compared to a healthy control group (data not shown).

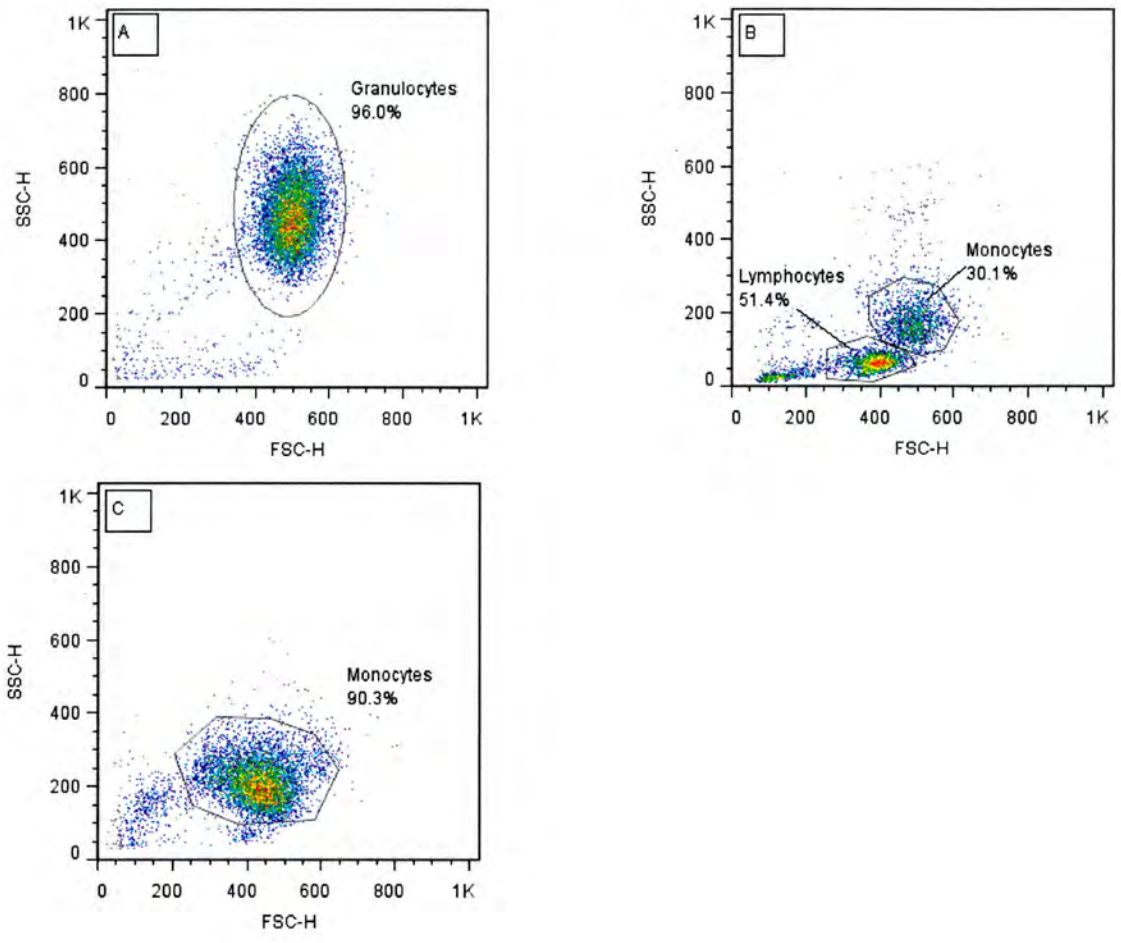


Figure 3.8 Flow Cytometry Check of Blood Preparation Purity. Neutrophils were isolated from whole blood using dextran sedimentation and Percoll extraction. Forward Scatter (FSC-H) and Side Scatter (SSC-H) represent cell size and granularity respectively. The gates were set on the established FSC-H and SSC-H positions of blood cells. [A] The granulocyte layer was shown on a cytospin preparation to consist of >99% neutrophils. [B] The Mononuclear layer consisted of predominately monocytes and lymphocytes seen on cytospins. [C] After $CD14^{+}$ magnetic bead labelling and passage through a magnetic column, monocyte purity was enhanced to >90%. (The above blood preparation was taken from a single representative patient).

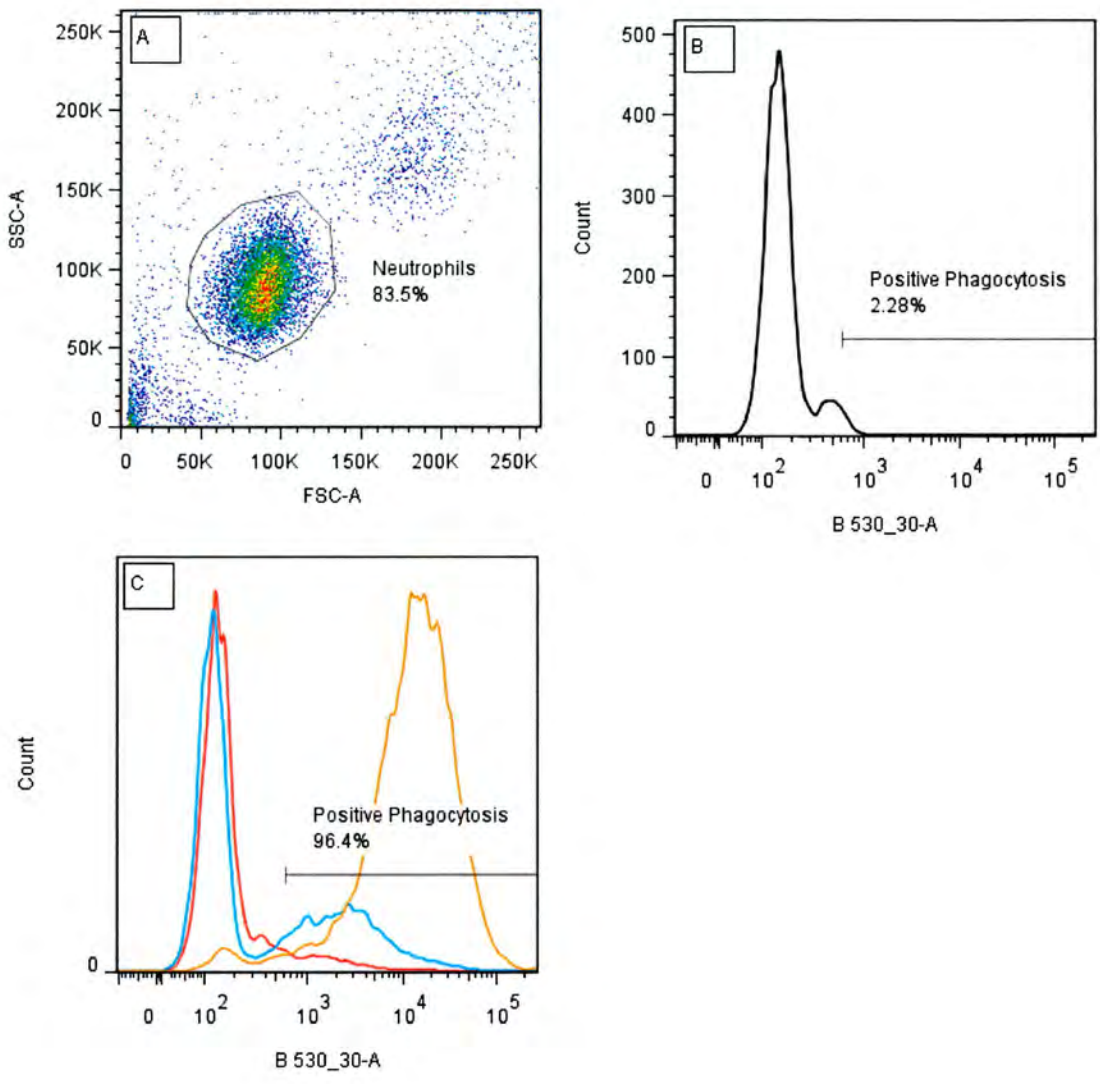
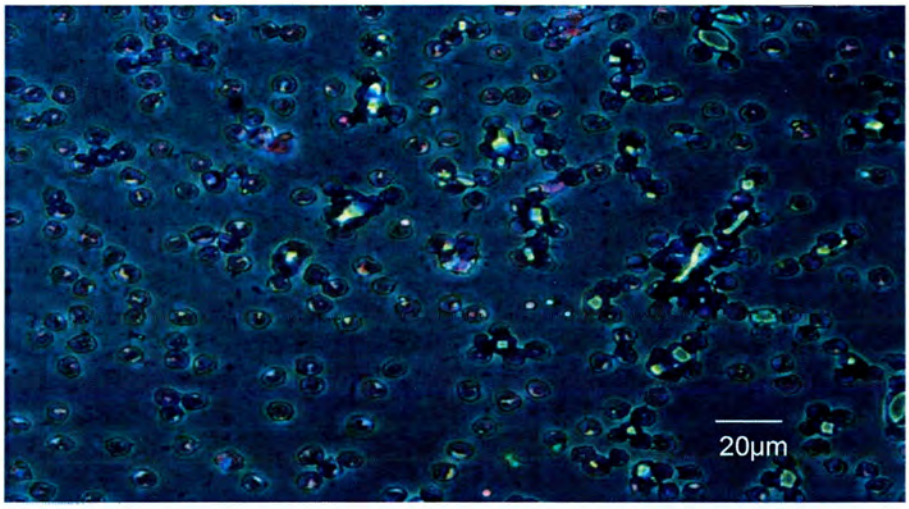
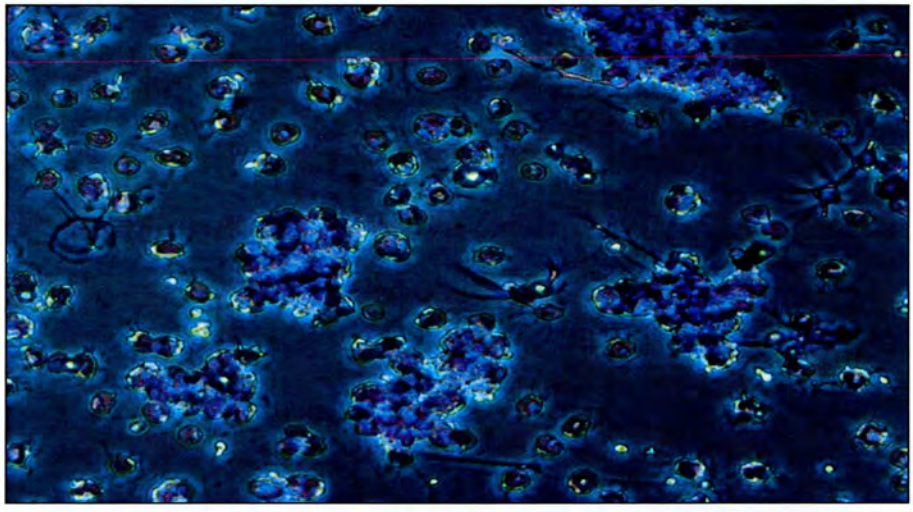


Figure 3.9 Flow Cytometry Analysis of Suspended Neutrophil Phagocytosis (*E. coli*). Isolated neutrophils were cultured with fluorescently labelled *E. coli*. After culture, typan blue was added and the cells washed before flow cytometry analysis. Forward Scatter (FSC-H) and Side Scatter (SSC-H) represent cell size and granularity respectively. B530_30-A is the channel detecting Alexa-Fluoro 488 which was pre-conjugated with *E.coli*. [A] The neutrophils were gated on according to typical FSC-H and SSC-A location. [B] A gate was placed for positive phagocytosis based on the autofluorescence of the unstained sample. [C] The positive phagocytosis gate was then applied to all the samples of the patient. Red=*E. coli* and no serum (Control), Blue=*E. coli* + serum (Cold) (Control), Orange=*E. Coli* and serum (96.4% positive). The above analysis was from a single representative patient. The same analysis was performed for Zymosan using the YG 610/20 channel.

[A]



[B]



[C]

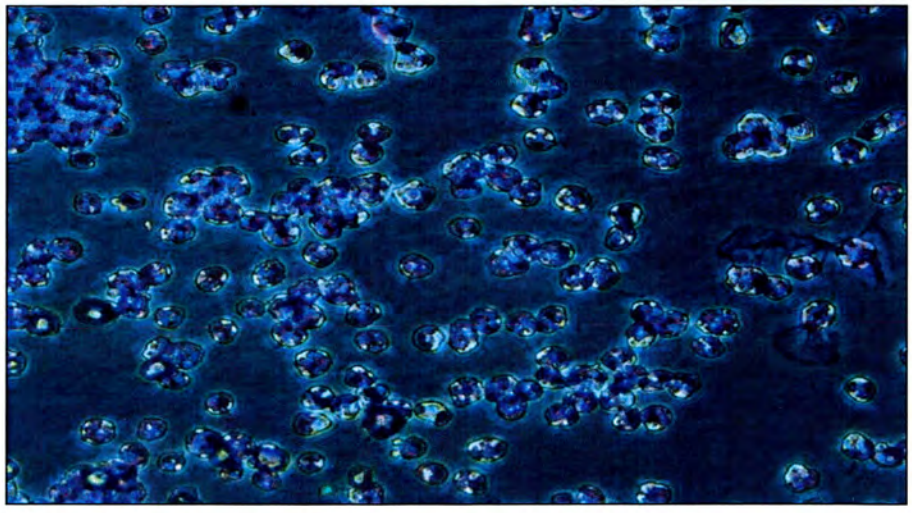


Figure 3.10 Light Microscopy Analysis of Plated Zymosan Phagocytosis. Images presented above. Neutrophils were separated from whole blood and cultured in individual wells of a 24-well Costar® plate for 30 minutes to enable cell adherence. Zymosan was then added for 30 minutes before washing and perfoming a Giemsa stain. [A] Plated neutrophils alone i.e. no zymosan or serum. [B] Plated neutrophils with non-opsonised zymosan i.e. no serum added (control). Note clumps of zymosan but few in the neutrophil cytoplasm. [C] Plated neutrophils with opsonised zymosan i.e. serum added. Note zymosan particles in the neutrophil cytoplasm. Cells with ≥2 zymosan particles were considered positive for phagocytosis. These images were from a single representative patient.

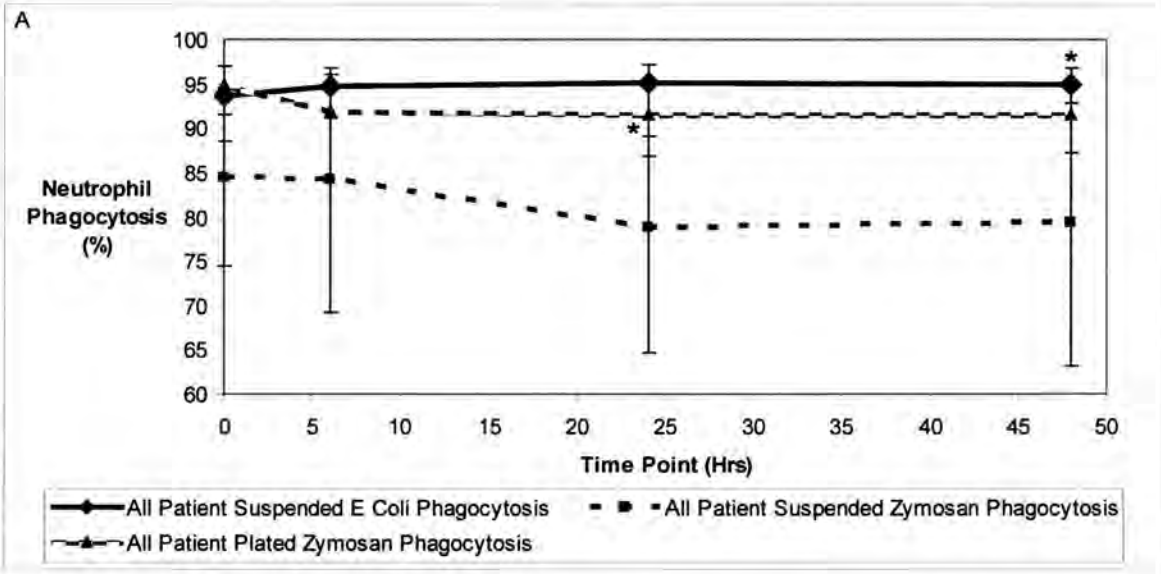
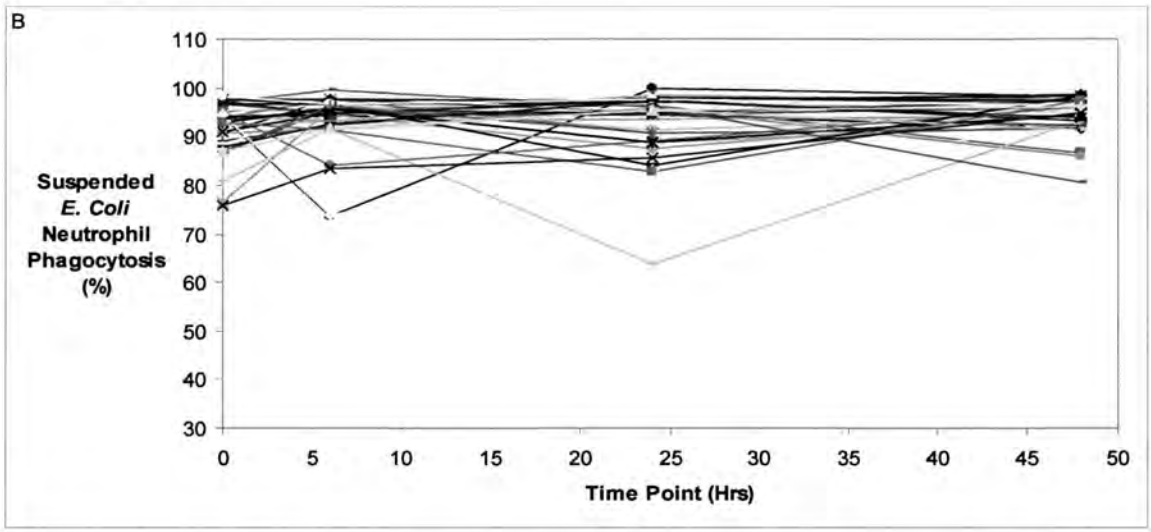
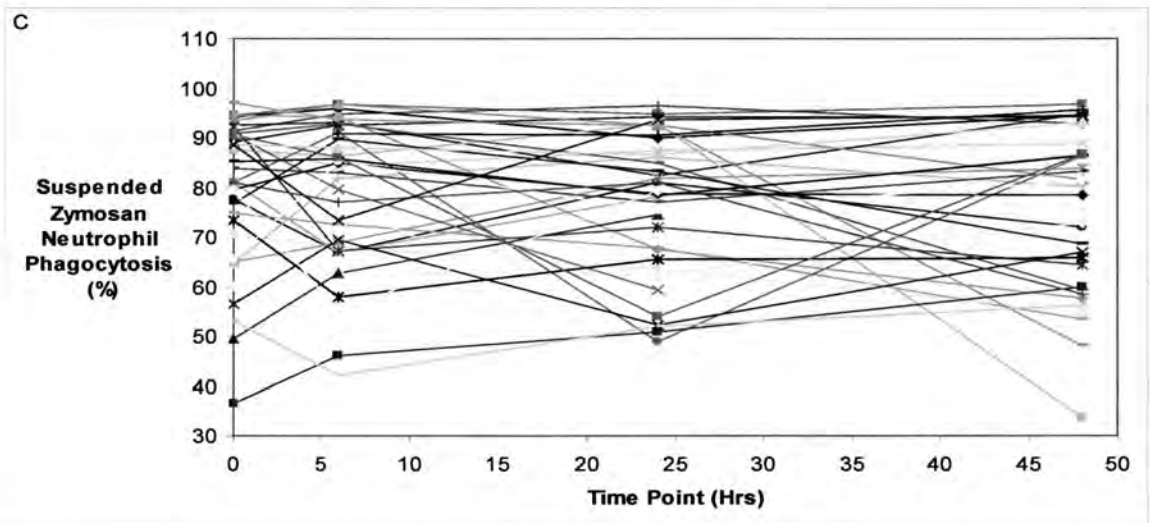
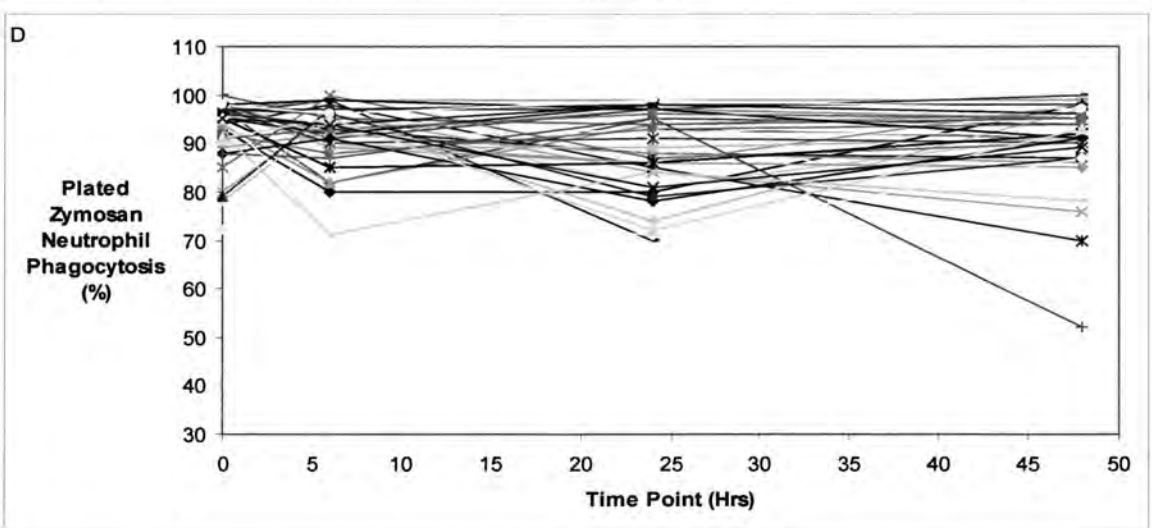


Figure 3.11 Summary of Post-Operative Trends in Neutrophil Phagocytosis. [A] The data was not normally distributed thus median values from each assay are shown with error bars representing the interquartile ranges. ANOVA modelling revealed a significant reduction in suspended Zymosan phagocytosis post-operatively (p=0.047) although no difference was found for *E. coli* (p=0.318) or plated Zymosan phagocytosis (p=0.080). Paired t-tests revealed a significant reduction in plated neutrophil Zymosan phagocytosis at 24 hrs (p=0.018) and an increase in *E. coli* phagocytosis at 48 hrs post-operatively (p=0.028) (denoted by *). [B-D] Trend lines for individual patients are shown below revealing *E.coli* and plated Zymosan phagocytosis to be generally high with an occasional outlier but much greater variability in suspended Zymosan phagocytosis. No significant differences between VATS and thoracotomy patients were found (p>0.05, data not shown). There was also no significant difference in baseline phagocytosis compared to a healthy control group n=10 (p>0.05, data not shown). n=37 for suspended *E.coli* phagocytosis, n=38 for suspended zymosan phagocytosis and n=40 for plated phagocytosis except n=38 at 48hrs.


B
Suspended E. Coli Neutrophil Phagocytosis (%)
110
100
90
80
70
60
50
40
30
0
5
10
15
20
25
30
35
40
45
50
Time Point (Hrs)


C
Suspended Zymosan Neutrophil Phagocytosis (%)
110
100
90
80
70
60
50
40
30
0
5
10
15
20
25
30
35
40
45
50
Time Point (Hrs)


D
Plated Zymosan Neutrophil Phagocytosis (%)
110
100
90
80
70
60
50
40
30
0
5
10
15
20
25
30
35
40
45
50
Time Point (Hrs)

Cytokine Responses after Monocyte Stimulation with LPS

Monocyte function was examined peri-operatively to determine whether a hyper- or hypo-inflammatory response was induced by lung resection. Monocytes were isolated from whole blood using dextran sedimentation, Percoll extraction and positively selected from the mononuclear layer using $CD14^+$ labelled magnetic beads and passage through a magnetic column. Purity following this process was assessed by flow cytometry as shown in Figure 3.8 [B&C]. Monocytes were then stimulated with LPS or no LPS (un-stimulated control).

Table 3.8 compares the cytokine response for the control patients and the pre-operative patient sample. In the un-stimulated control for both groups, low levels of cytokine were produced with the notable exception of IL-8. LPS induced a pro-inflammatory response from monocytes with increased levels of IL-8, IL-6, IL-1β, TNF-α and IL-10. There was no change in IL-12 levels following LPS stimulation. There was no statistically significant difference in cytokine response after LPS stimulation between the pre-operative patient samples and healthy control patients.

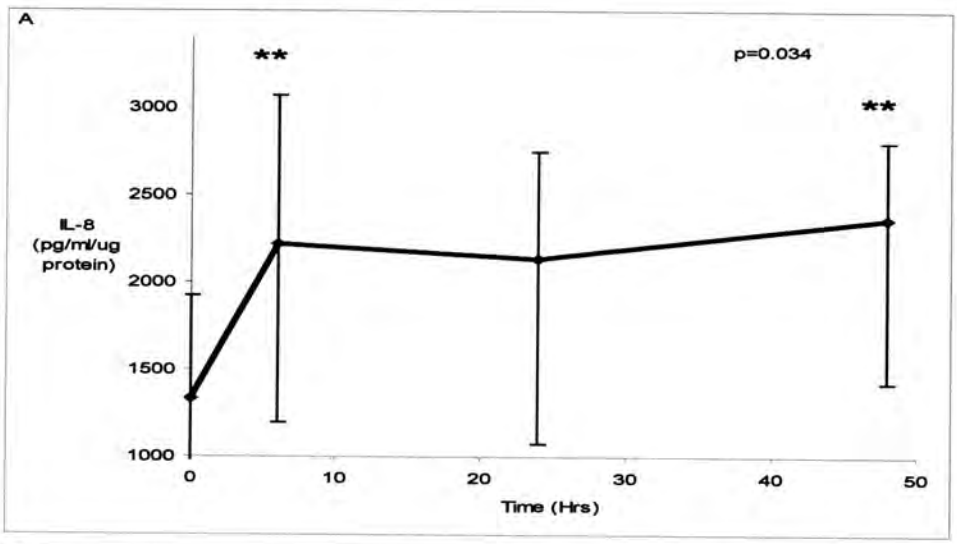
Figure 3.12 compares the patient monocyte response to LPS across the four time points. Monocytes produced relatively more IL-8, IL-6 and TNF-α post-operatively which reached statistical significance at 48 hrs (IL-8 geometric mean ratio 1.43 95% CI 1.10 - 1.85 p=0.007, IL-6 geometric mean ratio 1.42 05% CI 1.04-1.94 p=0.028, TNF-α geometric mean ratio 1.34 95% CI 1.02-1.74 p=0.034). There was also a significant increase for IL-8 at 6hrs (geometric mean ratio 1.48 95% CI 1.18-1.85 p=0.001). IL-10 levels fell post-operatively reaching significance at 48hrs (geometric mean ratio 1.45 95% CI 1.01-1.91 p=0.010). There was no significant change for IL-12 or IL-1β. ANOVA modelling revealed a significant post-operative change for TNF-α (p=0.028) and IL-8 (p=0.034) alone.

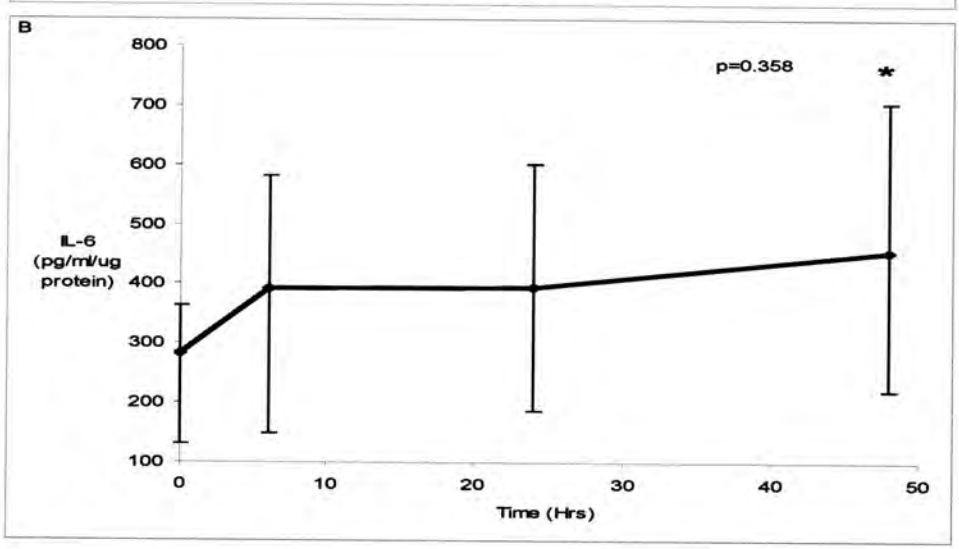
No statistically significant difference was found when comparing VATS versus thoracotomy patients on ANOVA modelling. Nevertheless, lower levels of IL-10 were produced by monocytes from VATS patients compared to thoracotomy at 6 hrs post-operatively (geometric mean ratio 1.16 95% CI 1.08-1.33 p=0.011, data not shown).

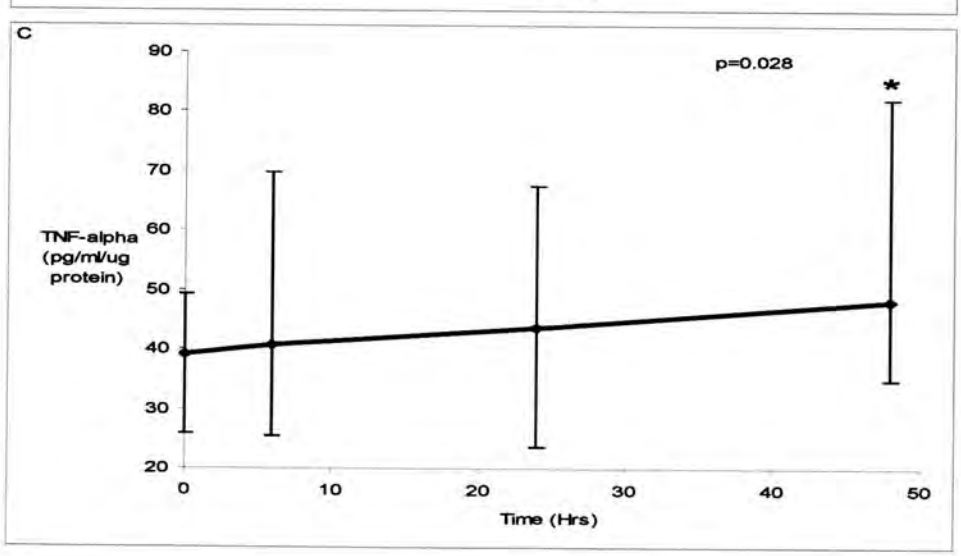
Cytokine	Control Patients Un-stimulated Median (n=10)	Surgical Patients Un-stimulated Median (n=40)	Control Patients LPS Median (n=10)	Surgical Patients LPS Median (n=40)	p-value LPS
IL-1β	15.2 (7.1-158.9)	1.6 (0.2-10.0)	69.2 (40.1-121.2)	28.1 (15.5-52.7)	**0.021**
IL-6	54.2 (13.7-159.7)	13.3 (4.3-158.9)	190.3 (64.1-252.5)	282.6 (130.3-362.7)	0.316
IL-8	1376.1 (667.2-1780.8)	826.5 (260.1-1660.9)	1217.6 (525.8-1353.0)	1334.0 (915.9-1976.2)	0.516
IL-10	0.4 (0.2-1.0)	0.20 (0.10-0.8)	1.5 (0.4-1.9)	2.1 (1.2-3.5)	0.622
IL-12	0.2 (0.2-0.2)	0.20 (0.1-0.2)	0.2 (0.2-0.2)	0.20 (0.1-0.3)	0.377
TNF-α	17.2 (2.1-31.3)	3.5 (0.3-33.3)	30.9 (23.0-42.9)	39.2 (25.9-49.6)	0.615

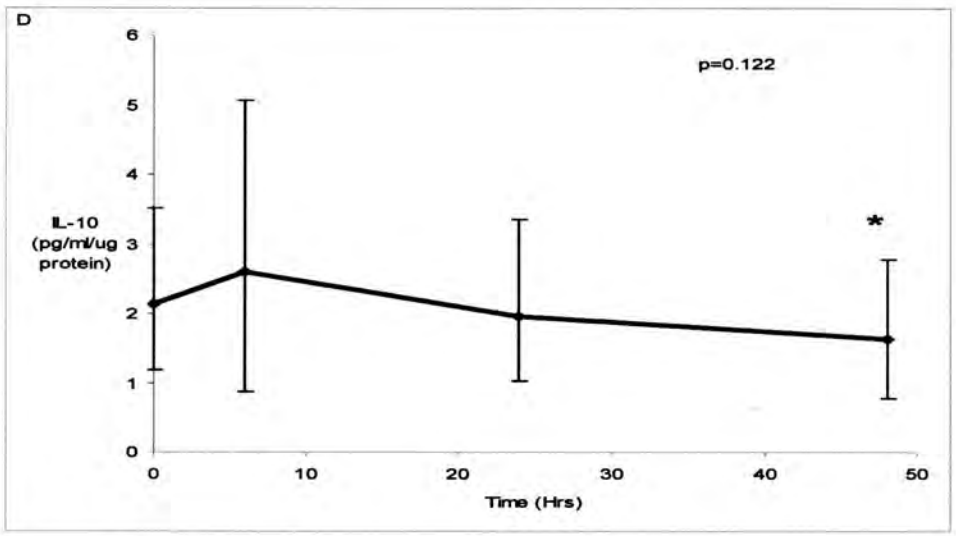
Table 3.8 Controls for Monocyte Response to LPS. The pre-operative surgical patients' monocyte response to normal culture and LPS stimulation is compared to that of a similar aged healthy control population. Median and interquartile ranges are shown due to not normal distribution of data. A significant response to IL-8 was notable in the un-stimulated cells. No significant difference in monocyte cytokine responsiveness to LPS was found except for IL-1β.

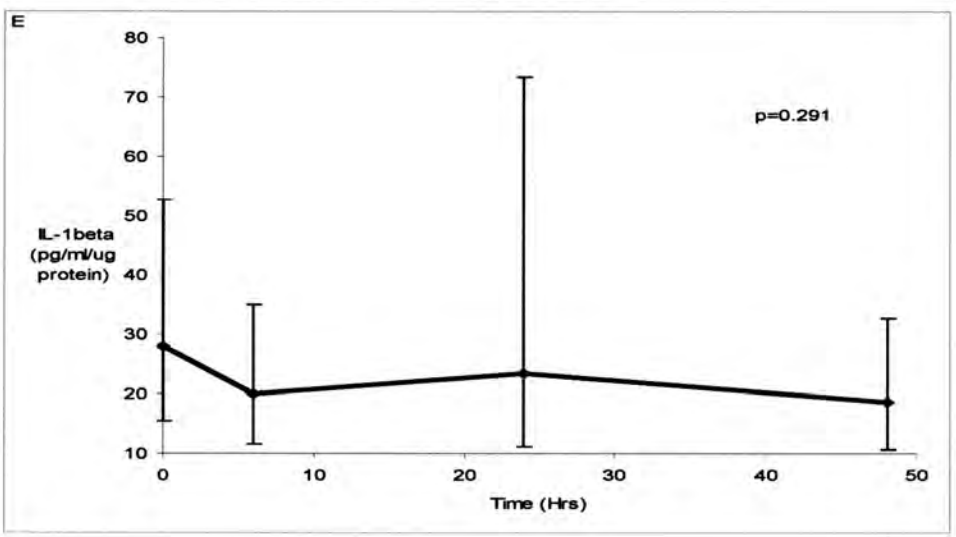
Figure 3.12 Ex-vivo Monocyte Cytokine Responsiveness to LPS Stimulation. Panels [A-F] shown below. Monocytes were isolated from whole blood and stimulated with LPS for 18 hrs and the supernatant analysed for cytokines (A, IL-8; B, IL-6; C, TNF-α; D, IL-10; E, IL-1β; F, IL-12). The data were adjusted for total protein content, and are presented as medians and interquartile ranges due to not normal distribution. Overall trends were analysed by ANOVA modelling (as reflected in the p value in each panel); individual time points were assessed relative to the pre-operative value by paired t-test with significant results denoted thus *, $p<0.05$, **, $p<0.005$. No significant differences were found when comparing VATS versus thoracotomy groups on ANOVA modelling. However, a two sample t-test showed that lower levels of IL-10 were produced by monocytes from VATS compared to thoracotomy patients at 6 hrs post-operatively [panel G]. n=40 at all time points except n=38 at 48hrs.

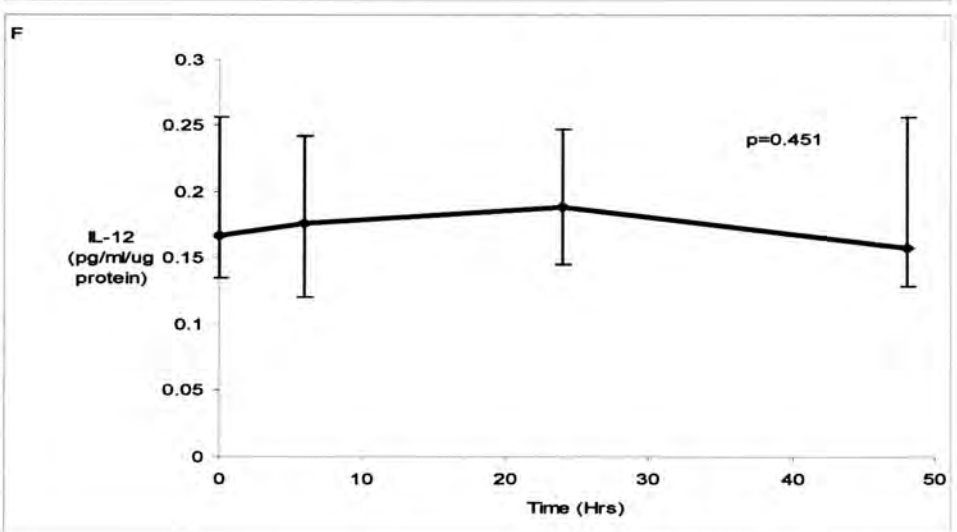

A
**
p=0.034
**
3000
2500
2000
1500
1000
IL-8
(pg/ml/ug
protein)
0
10
20
30
40
50
Time (Hrs)

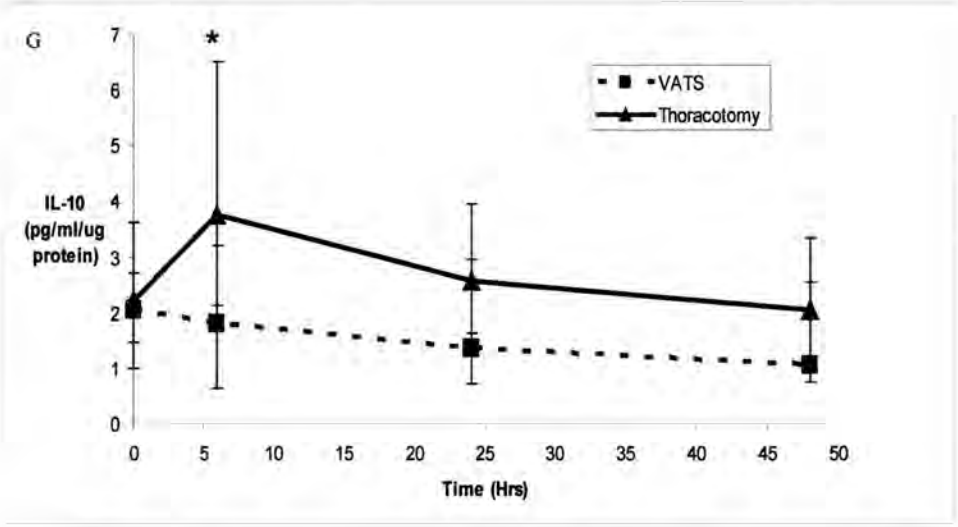

B
800
700
600
500
400
300
200
100
p=0.358
*
IL-6
(pg/ml/ug
protein)
0
10
20
30
40
50
Time (Hrs)


C
90
80
70
60
50
40
30
20
p=0.028
*
TNF-alpha
(pg/ml/ug
protein)
0
10
20
30
40
50
Time (Hrs)


D
p=0.122
IL-10 (pg/ml/ug protein)
*
Time (Hrs)


E
p=0.291
IL-1beta (pg/ml/ug protein)
Time (Hrs)


F
p=0.451
IL-12 (pg/ml/ug protein)
Time (Hrs)



Monocyte Subset Changes

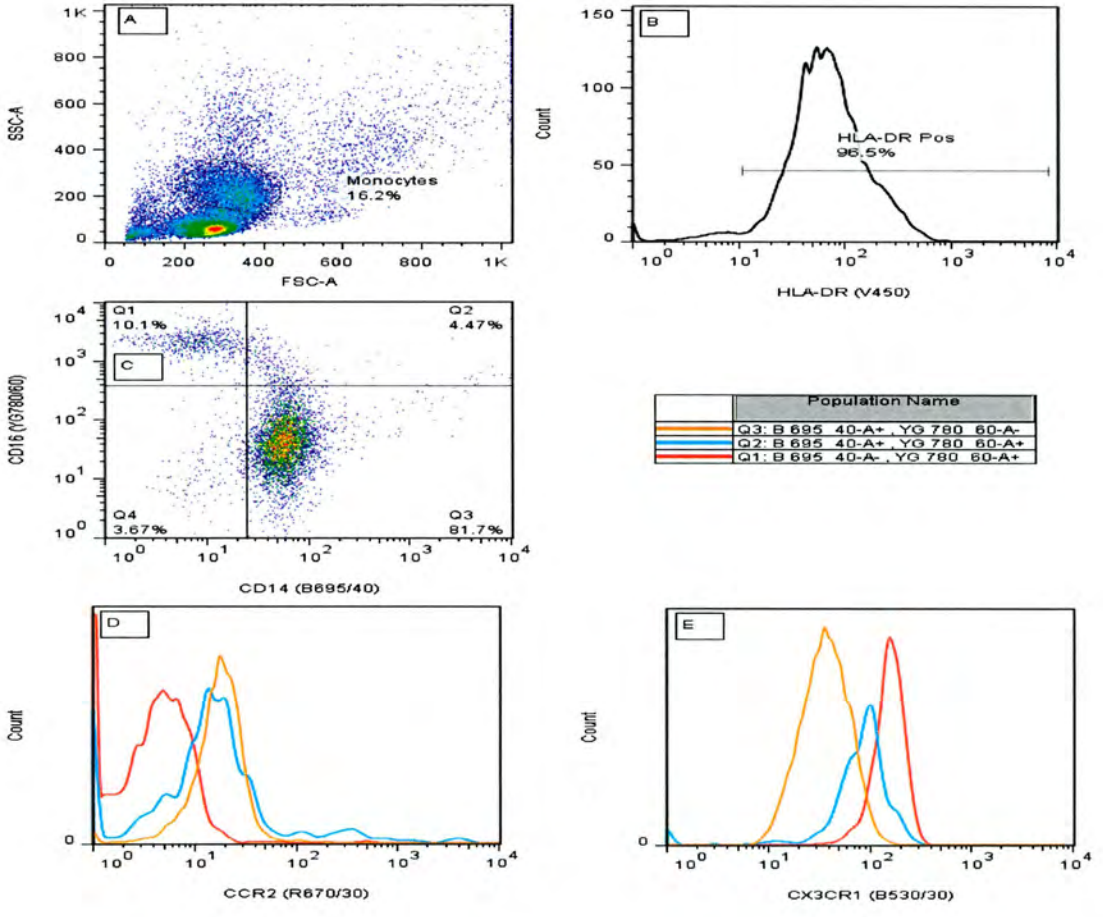
The existence of different populations of human monocytes is well established (Ziegler-Heitbrock et al., 2010). The classical monocytes ($CD14^{++}$ $CD16^{-}$) form the majority of circulating blood monocytes. However, a non-classical ($CD14^{+}CD16^{++}$) subset has also been identified with higher cytokine production and antigen presentation together with an intermediate subset ($CD14^{++}$ $CD16^{+}$). The three principal monocyte subsets were identified using five-colour flow cytometry analysis. Figure 3.13 describes the flow cytometry analysis. The test tube with all five antibodies was used to compare monocyte subset changes between the time points for each patient. Figure 3.14 illustrates the typical post-operative monocyte subset changes as determined on flow cytometry.

Table 3.9 summarises the relative change in the three monocyte subsets post-operatively for all patients. There was a statistically significant relative reduction in non-classical monocytes post-operatively. This reduction was maximal at 6hrs post-operatively (geometric mean ratio 2.6 95% CI 1.65-4.09 $p<0.0005$) which increased at 24hrs (geometric mean ratio 1.64 95% CI 1.17-2.3 $p=0.005$) before falling again at 48hrs

post-operatively (geometric mean ratio 2.04 95% CI 1.42-2.92 $p<0.0005$). There was no significant relative change for the other monocyte subsets. There was no significant difference found for VATS compared with thoracotomy patients.

However, calculation of the absolute number of each monocyte subset, taking into account the overall monocyte count, revealed a significant increase in intermediate and classical monocytes which caused this relative reduction in non-classical monocytes (Table 3.10).

Figure 3.13 Flow Cytometry analysis of monocyte subsets. Mononuclear cells were isolated from whole blood using dextran sedimentation and Percoll extraction. Five fluorescently conjugated antibodies were used to identify monocyte subsets (CD14, CD16, HLA-DR, CX_3CR1, CCR2). An unstained sample and five single stains were used to compensate the data with a mixture sample containing all five antibodies used to identify monocyte subsets. The figure below demonstrates the five-colour flow cytometry analysis from a representative patient at one time point. [A] Monocytes were identified based on typical size and granularity (forward scatter (FSC-H) and side scatter (SSC-H) respectively). [B] Cells positive for HLA-DR staining were selected. [C] Three monocyte populations were identified based on $CD14^+$ and $CD16^+$ staining. [D&E] CCR2 and CX_3CR1 staining confirmed correct identification of monocyte subsets: Q1- $CD14^-$ $CD16^{++}$ $CX_3CR1^{++}CCR2^-$; Q2- $CD14^{++}$ $CD16^+CCR2^+$ $CX3CR1^+$; Q3-$CD14^{++}$ $CD16^-$ $CCR2^{++}$ $CX3CR1^-$.


A
SSC-A
FSC-A
Monocytes
16.2%
B
Count
HLA-DR Pos
96.5%
HLA-DR (V450)
C
CD16 (V0780/60)
CD14 (B695/40)
Q1
10.1%
Q2
4.47%
Q4
3.67%
Q3
81.7%
Population Name
Q3: B 695 40-A+ , YG 780 60-A-
Q2: B 695 40-A+ , YG 780 60-A+
Q1: B 695 40-A- , YG 780 60-A+
D
Count
CCR2 (R670/30)
E
Count
CX3CR1 (B530/30)

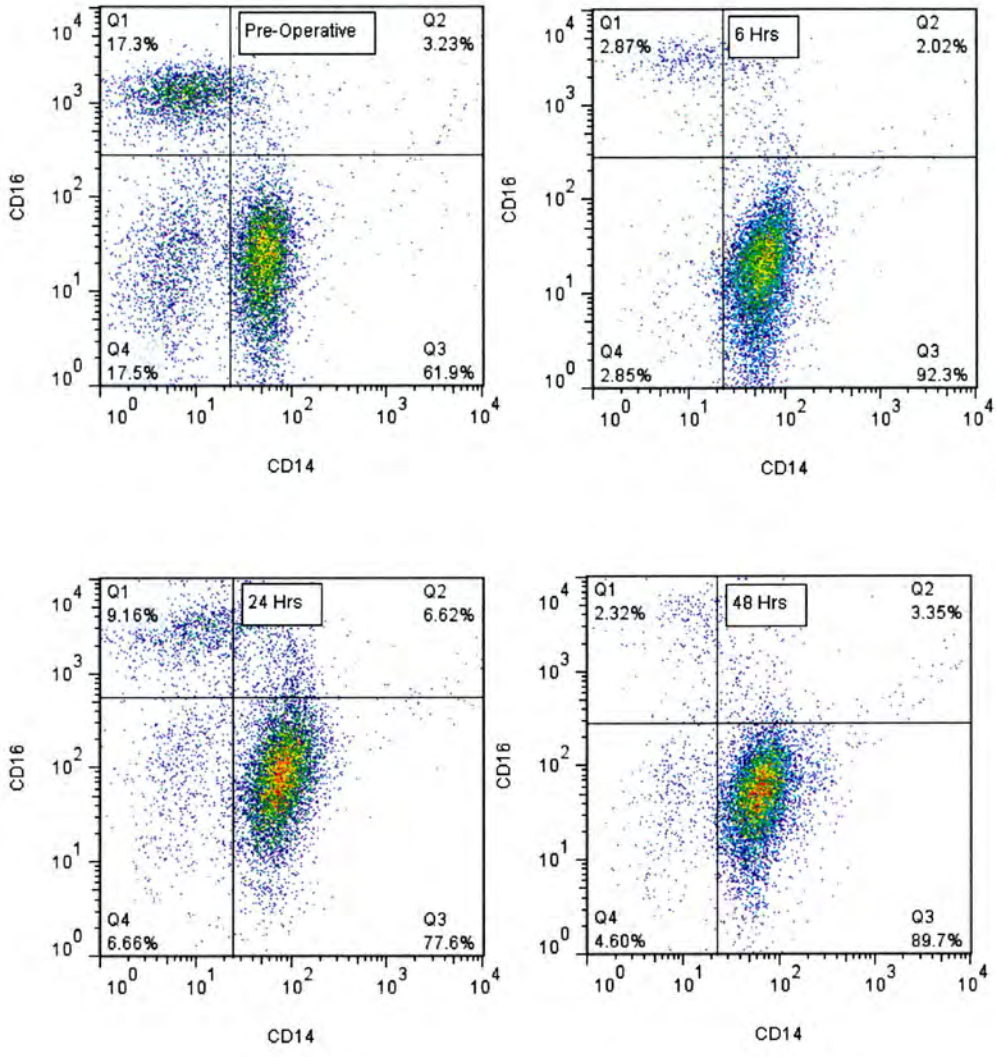


Figure 3.14 Monocyte Subset Changes Following Lung Resection. Monocytes were labelled and analysed as described in Figure 3.13. The relative post-operative changes in the three monocyte subsets are illustrated in the flow cytometry quadrants above (obtained from a single representative patient). The percentage of cells in each of the four quadrants was used to calculate the relative change in subsets and, in turn, the absolute change proportionate to the total monocyte count.

Monocyte Subset	Pre-Op Median (%)	6Hrs Median (%)	24Hrs Median (%)	48Hrs Median (%)	p-value 6Hrs	p-value 24Hrs	p-value 48Hrs
$CD14^{+}CD16^{++}$ (Q1)	5.5 (2.7-10.0)	3.0 (1.5-4.0)	3.1 (1.9-5.3)	2.5 (1.9-4.1)	**<0.0005**	**0.005**	**<0.0005**
$CD14^{++}CD16^{+}$ (Q2)	3.8 (2.8-5.1)	2.9 (2.2-5.2)	5.3 (3.2-7.2)	4.3 (3.5-6.4)	0.086	0.121	0.072
$CD14^{++}CD16^{-}$ (Q3)	80.9 (73.3-86.5)	89.2 (86.3-92.4)	85.8 (79.8-89.3)	87.0 (82.5-90.4)	0.072	0.129	0.118

Table 3.9 Relative Post-Operative Change in Monocyte Subsets. The median percentage of monocytes in each subset at each time point is shown above. Figures 3.13 and 3.14 detail the underlying flow cytometry analysis. The data was not normally distributed as determined by the Anderson-Darling test requiring $\log_{10}$ transformation prior to further analysis. Paired t-tests were used to determine post-operative change compared to the pre-operative value. Significant values are shown in bold ($p<0.05$). n=39 for all time points except n=37 at 48hrs.

Monocyte Subset	Pre-Op Median Count ($x10^9/l$)	24Hrs Median Count ($x10^9/l$)	48Hrs Median Count ($x10^9/l$)	p-value 24Hrs	p-value 48Hrs
$CD14^{+}CD16^{++}$ (Q1)	0.04 (0.02-0.06)	0.03 (0.02-0.04)	0.02 (0.02-0.04)	0.286	0.057
$CD14^{++}CD16^{+}$ (Q2)	0.02 (0.02-0.04)	0.04 (0.03-0.06)	0.04 (0.03-0.06)	**0.001**	**<0.0005**
$CD14^{++}CD16^{-}$ (Q3)	0.53 (0.45-0.66)	0.76 (0.63-0.90)	0.79 (0.62-1.03)	**0.001**	**0.008**

Table 3.10 Absolute Post-Operative Changes in Monocyte Subset Counts. The absolute changes in the monocyte subset counts were calculated from the relative percentage change as a proportion of total blood monocyte count ($x10^9/l$). The median monocyte subset count at each time point is shown above. There was no overall monocyte count conducted at 6hrs post-operatively. The data was not normally distributed as determined by the Anderson-Darling test requiring $\log_{10}$ transformation prior to further analysis. Paired t-tests were used to determine post-operative change compared to the pre-operative value. Significant values are shown in bold ($p<0.05$). n=39 pre-op, n=38 24hrs and n=27 48hrs.

BAL Cell Concentration, Total Protein and Cytokine Response After Lung Resection

BAL was used as a means of assessing the early impact of surgical trauma on the non-operated, comparatively healthy lung. A different lobe of the contra-lateral lung to the culprit lesion was sampled pre-operatively and immediately post-operatively prior to extubation. Table 3.6 details the lobes sampled from each patient and the volume of lavage fluid returned to the suction trap. The median return was 43mls pre-operatively (21.5%) and 45mls post-operatively (22.5%). No statistically significant difference was found in return volume between the time points (p=0.243).

The mean concentration of cells (millions/ml) in the BAL fluid increased post-operatively (Figure 3.15). The geometric mean ratio between pre-operative and post-operative BAL was 0.57 (95% CI 0.28-0.37 p=0.012). Differential cell count revealed an increase in neutrophils post-operatively however this was not statistically significant (Table 3.12). The total protein (μg/ml) also increased post-operatively with a geometric mean ratio of 0.61 (95% CI 0.40-0.95 p=0.029) (Figure 3.16). There was no significant difference for VATS versus thoracotomy patients for cell concentration, differential cell count and total protein using Paired T-tests and ANCOVA modelling.

Surgery induced an early cytokine response as detailed in Figure 3.17. There was a post-operative increase in IL-6 (geometric mean ratio 0.40 95% CI 0.26-0.61 p<0.0005), IL-8 (geometric mean ratio 0.49 95% CI 0.28-0.87 p=0.017) and IL-10 (geometric mean ratio 0.80 95% CI 0.66-0.96 p=0.018). However, there was an additional increase in IL-1β (geometric mean ratio 0.48 95% CI 0.30-0.75 p=0.002) in the BAL which was not found in the serum 6hrs later. There was no significant change in TNF-α or IL-12 (median value =0, data not shown). ANCOVA modelling or paired t-tests did not reveal any difference for VATS versus thoracotomy patients.

Location of Lesion	Lobe Lavaged Pre-Operative	Return Volume (mls)/200mls	Lobe Lavaged Post-Operative	Return Volume (mls)/200mls
LUL	RLL	51	RML	55
LUL	RLL	47	RML	25
RUL	No scope		No scope	
RML	LLL	45	LUL	65
LLL	RLL	43	RML	55
LUL	RLL	53	RML	35
LUL	RLL	55	RML	18
LUL	RLL	39	RML	19
RML	LLL	52	Lingula	45
LUL	RLL	45	RML	65
LUL	RLL	97	RML	22
LUL	RLL	40	RML	35
LUL	RLL	38	RML	70
RLL	LLL	55	Lingula	23
LUL	RLL	100	RML	55
RLL	LLL	45	Lingula	62
LUL	RLL	55	RML	8
LLL&LUL	RLL	60	RML	70
LUL	RLL	44	RML	75
RUL	LLL	41	Lingula	75
RUL	LLL	25	Lingula	30
LUL	RLL	15	RML	20
RML&RLL	LLL	45	Lingula	62
L Hilum/LUL	RLL	60	Nil - Pneumonectomy	
RUL	LLL	35	Lingula	27
RUL	LLL	30	Lingula	9
RUL	LLL	19	No scope	
RUL	LLL	42	Lingula	52
RUL	LLL	32	Lingula	50
RLL	LLL	32	Lingula	50
RUL	LLL	38	Lingula	20
LUL	RLL	74	RML	40
RUL	LLL	24	Nil - Pneumonectomy	
RML	LLL	30	Lingula	40
LUL	RLL	33	RML	45
RUL	LLL	54	Lingula	40
RML	LLL	38	Lingula	44
LUL	RLL	38	RML	60
RUL	LLL	58	Lingula	54
RML&RLL	LLL	42	Lingula	55
Median Return		**43 (36.5-53.5)** **21.5%**		**45 (26.5-56.3)** **22.5%**

Table 3.11 Summary of BAL Sampling Location and Volume Returned. BAL was undertaken after intubation for bronchoscopy and mediastinoscopy (pre-operative) and after lung resection prior to extubation (post-operative). A different lobe of the contra-lateral lung to

the culprit lesion was sampled on each occasion. A total of 200mls of 0.9% NaCl (in four 50ml aliquots) was injected and the total volume returned shown. The data was not normally distributed as determined by the Anderson-Darling test. A median value and interquartile range is shown for each time point. A Paired t-test was undertaken after $\log_{10}$ transformation revealing no statistically difference in return volume between the two time points (p=0.243).

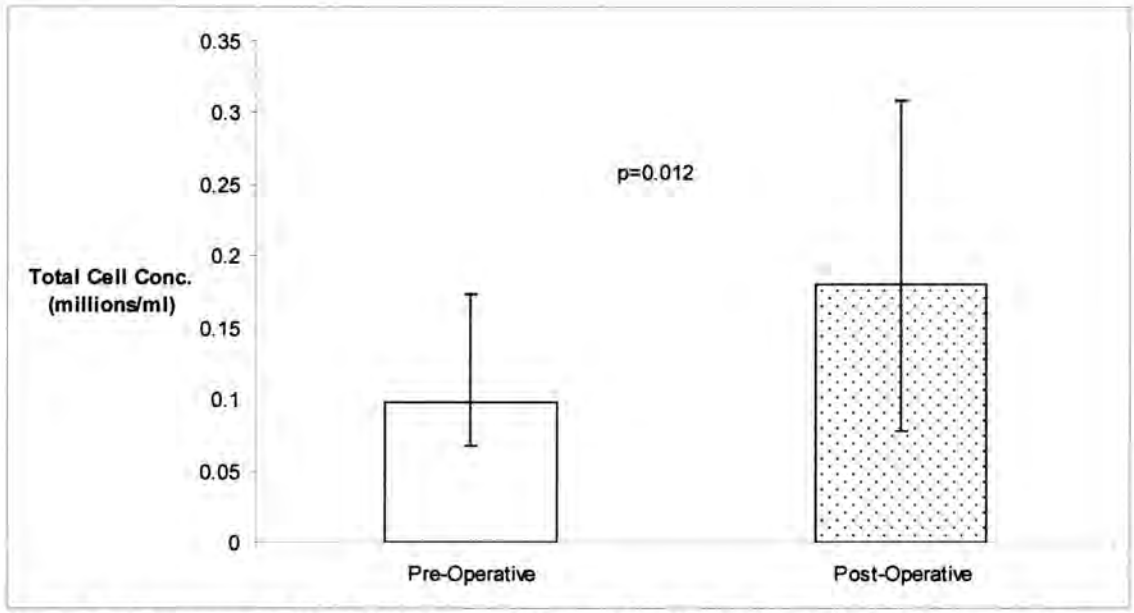


Figure 3.15 Increase in BAL Cell Concentration After Lung Resection. BAL was performed pre-operatively and post-operatively. On both occasions the non-operated lung was lavaged, and a different segment was sampled in the pre- and post-operative procedures. The data were not normally distributed and thus presented as median values with error bars representing the interquartile range. A paired T-test was performed after $\log_{10}$ transformation. Geometric mean pre-operatively was 0.09 which increased to 0.16 post-operatively (p=0.012). No significant difference between VATS and thoracotomy patients was found using paired t-tests and ANCOVA modelling (p>0.05, data not shown). n=36.

Cell Type	Pre-Operative Median (IQR)	Post-Operative Median (IQR)	p-value
Macrophages/Monocytes	93 (75-96)	84 (45-92)	0.232
Neutrophils	4 (2-23.5)	13 (3-49)	0.176
Lymphocytes	2 (1-3)	3 (1-4)	0.259
Other	<1% (0)	<1% (0)	1.000

Table 3.12 Changes in BAL Differential Cell Count Following Lung Resection. A different lobe of the contra-lateral lung to the culprit lesion was sampled on each occasion. A cytospin was performed of the pellet of cells obtained after centrifugation. Differential cell count was undertaken after Giemsa staining. The relative change in cell type counts were compared between the two time points. The data were not normally distributed, as determined by the Anderson-Darling test. Median and interquartile ranges are shown. Log_{10} transformation was performed prior to undertaking a Paired t-test. No statistically significant differences were found ($p<0.05$). n=33.

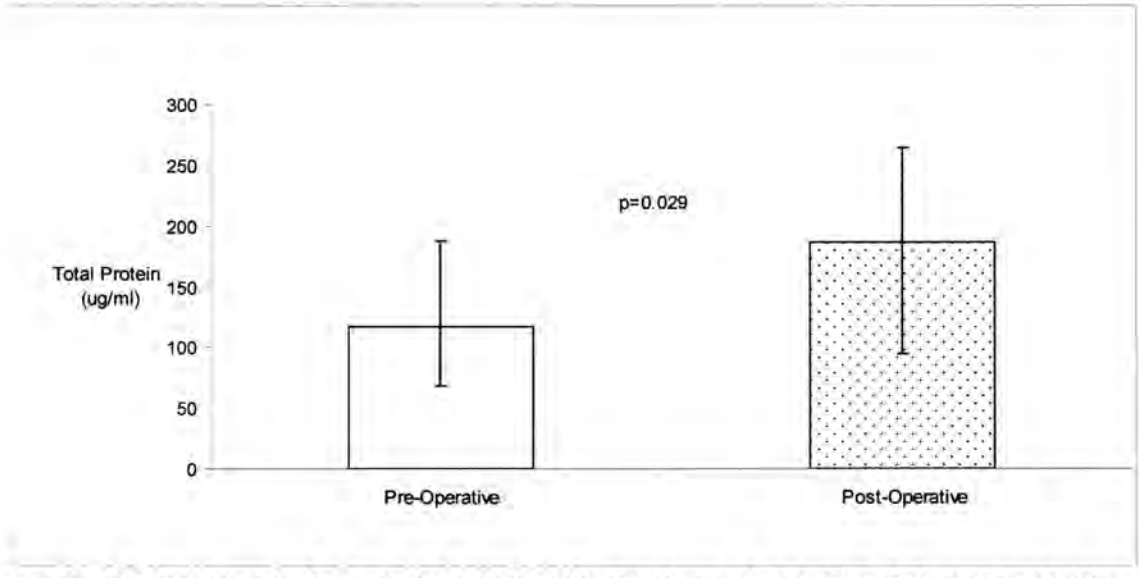


Figure 3.16 Increase in BAL Total Protein Following Lung Resection. BAL was undertaken prior to surgical staging from the contralateral lung to the culprit lesion and from another lobe of the contra-lateral lung after completion of lung resection. Pierce assay was used to assess total protein levels in BAL supernatant and was reported in µg/ml. The data were not normally distributed. Median values and interquartile ranges are shown. After log_{10} transformation a paired t-test was performed. Geometric mean pre-operatively was 97.07 which increased to 158.12 ($p=0.029$). No significant difference between VATS and thoracotomy patients was found using paired t-tests and ANCOVA modelling (data not shown). n=33.

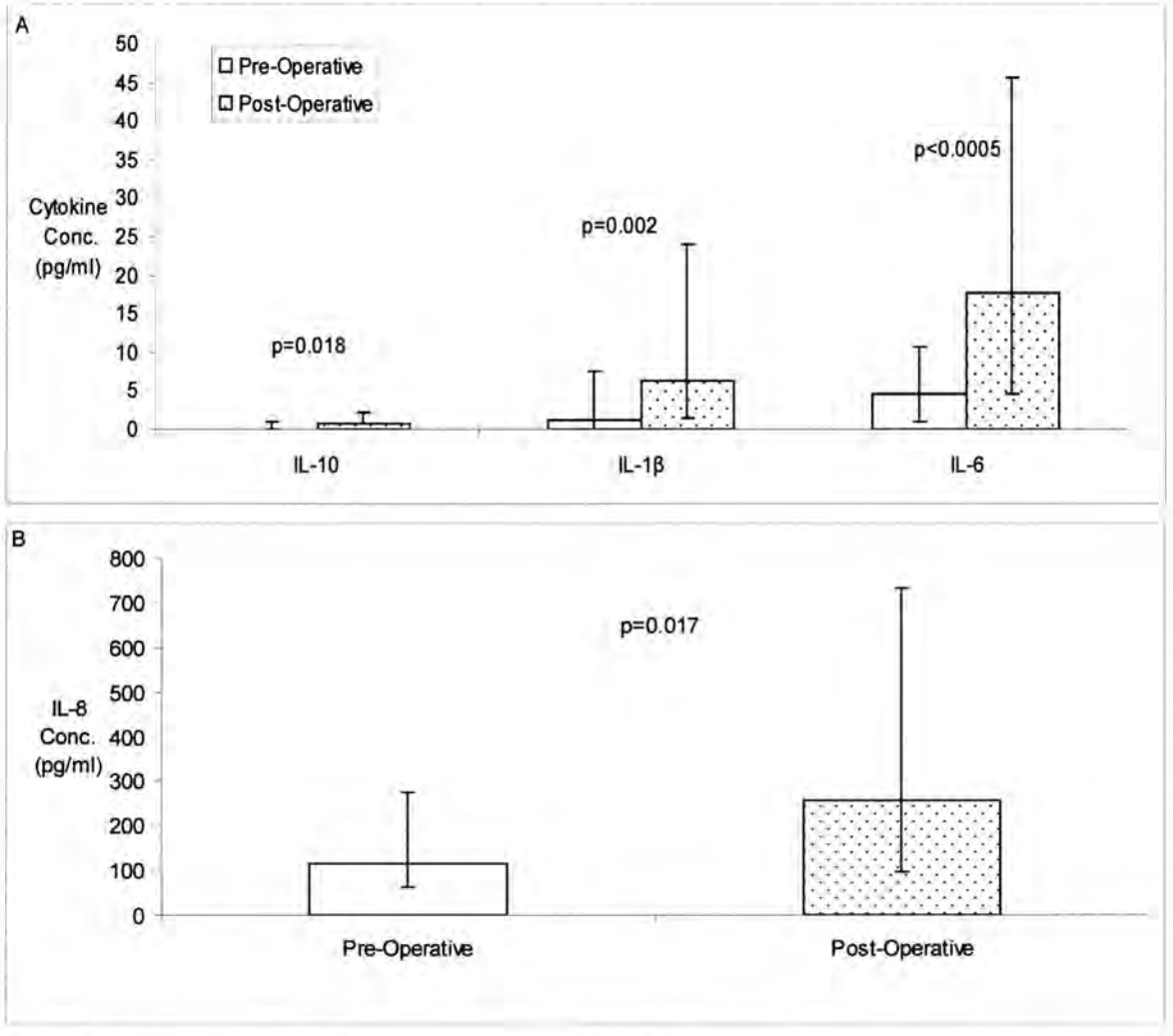
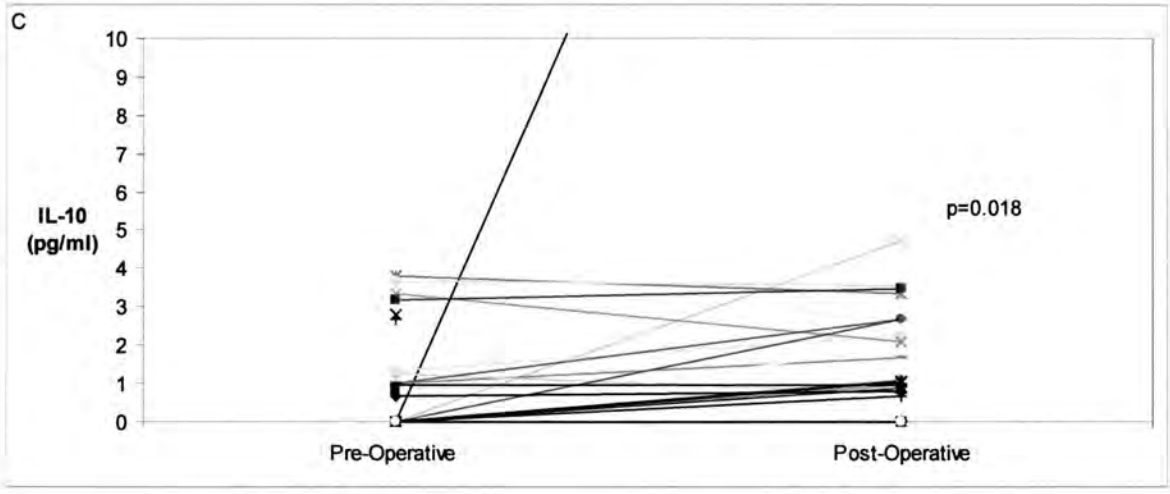
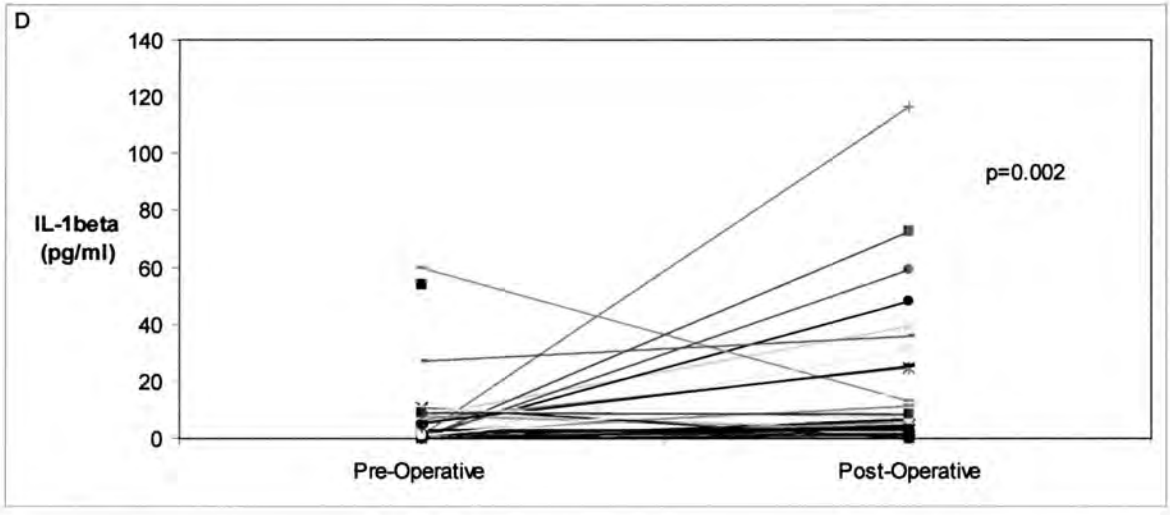
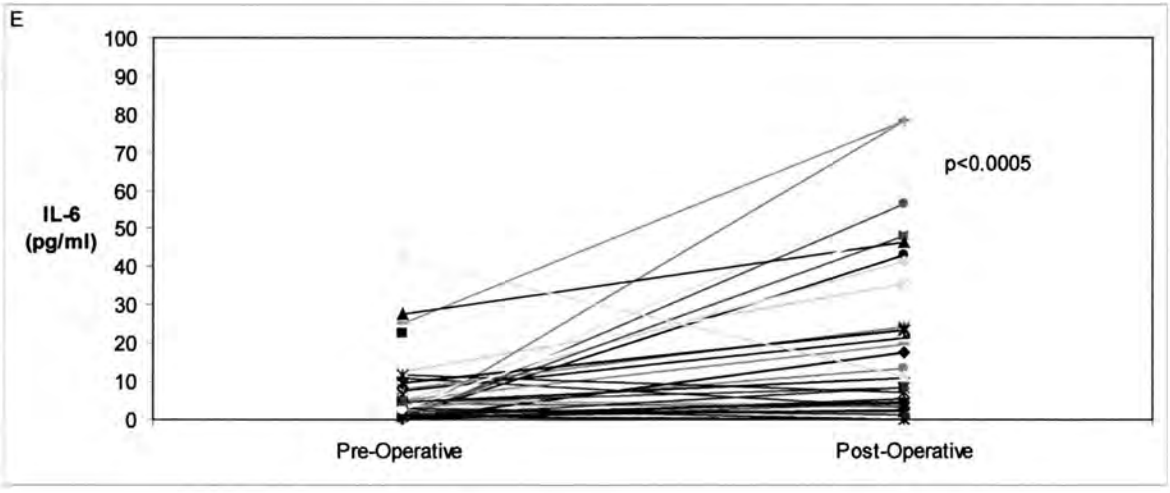
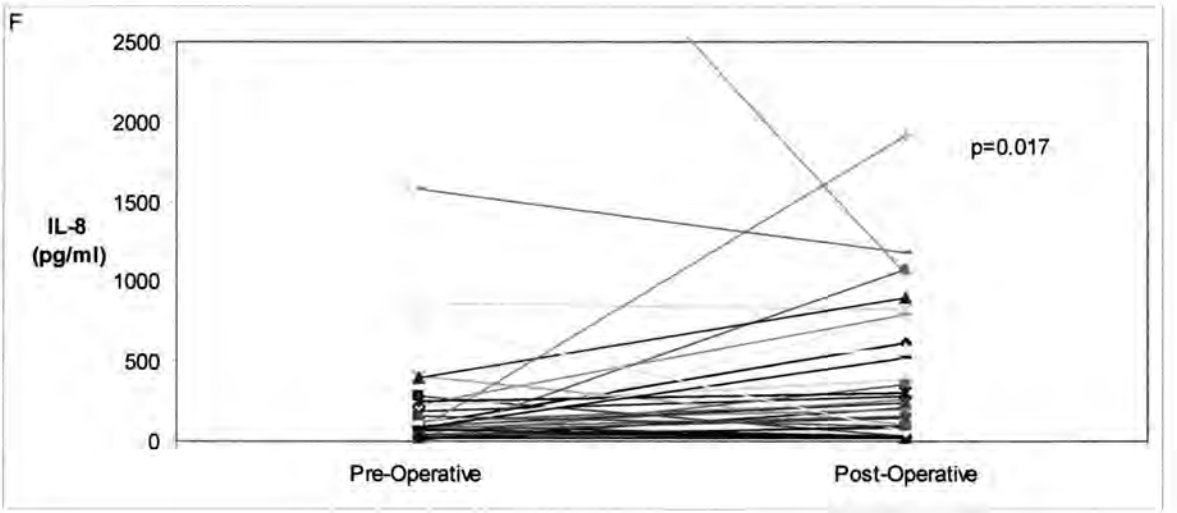


Figure 3.17 Post-Operative Increase in BAL Cytokines. BAL was undertaken prior to surgical staging from the contralateral lung to the culprit lesion and from another lobe of the contralateral lung immediately after completion of lung resection. [A&B] Cytokines in BAL supernatant were measured and required $\log_{10}$ transformation due to not normal distribution prior to undertaking a paired T-test. The median values at each time point and p-values are shown in the chart. Error bars represent the interquartile range. There was no significant difference in IL-12 or TNF-α (median value at each time point =0, data not shown). ANCOVA modelling did not reveal a difference between VATS and thoracotomy. [C-F] The individual patient post-operative trends for IL-10, IL-1β, IL-6 and IL-8 are shown below. n=35.


C
IL-10 (pg/ml)
10
9
8
7
6
5
4
3
2
1
0
p=0.018
Pre-Operative
Post-Operative


D
IL-1beta (pg/ml)
140
120
100
80
60
40
20
0
p=0.002
Pre-Operative
Post-Operative


E
IL-6 (pg/ml)
100
90
80
70
60
50
40
30
20
10
0
p<0.0005
Pre-Operative
Post-Operative



Exhaled Nitric Oxide

In an effort to identify a non-invasive measure of airway inflammation after lung resection, exhaled nitric oxide was evaluated. FENO (Fractional exhaled nitric oxide) had previously been shown to be a useful measure of airway inflammation in asthmatic patients. In this study, FENO was measured using the gold standard chemiluminescence analyser (Niox Flex) and the smaller electrochemical sensor (Niox Mino) at each time point. Both were set at flow rate 50mls/sec. Figure 3.18 demonstrates a strong positive correlation between the two methods ($r=0.902$, $p<0.0005$). In addition, the Altman-Bland plot suggested a high degree of agreement between the devices (Figure 3.19).

Figure 3.20 depicts the post-operative trend in exhaled nitric oxide. Importantly, not all patients could perform all of the post-operative measurements thus the paired t-test resulted in slightly different pre-operative mean values for each post-operative time point. There was an overall tendency towards reduction in exhaled nitric oxide post-operatively which reached statistical significance at 48hrs (geometric mean ratio 1.2 95% CI 1.02-1.46 $p=0.029$). There was no significant difference found between patients undergoing resection via VATS or thoracotomy ($p>0.05$, data not shown).

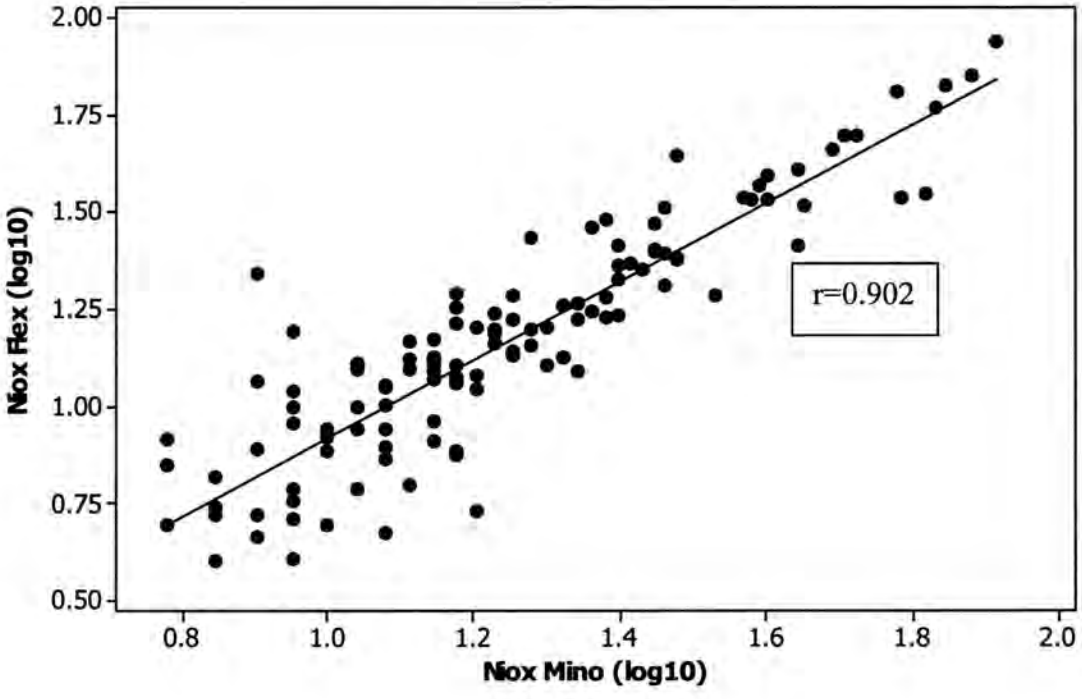


Figure 3.18 Comparison of Exhaled Nitric Oxide (FENO) Measurements from Niox Flex and Niox Mino Devices. FENO readings in parts per billion (ppb) were not normally distributed thus $\log_{10}$ transformed for the scatter plot. Pearson correlation coefficient confirmed a strong positive association r=0.902 ($p<0.0005$).

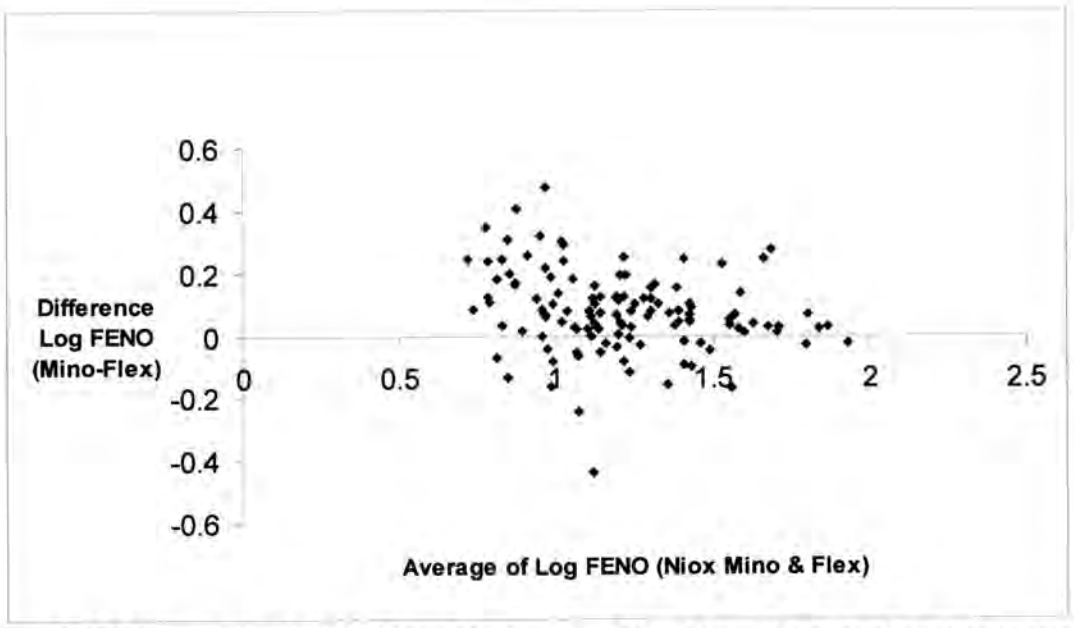


Figure 3.19 Altman-Bland plot of Niox Mino versus Niox Flex. The above figure suggests a high degree of agreement in measurements between the devices.

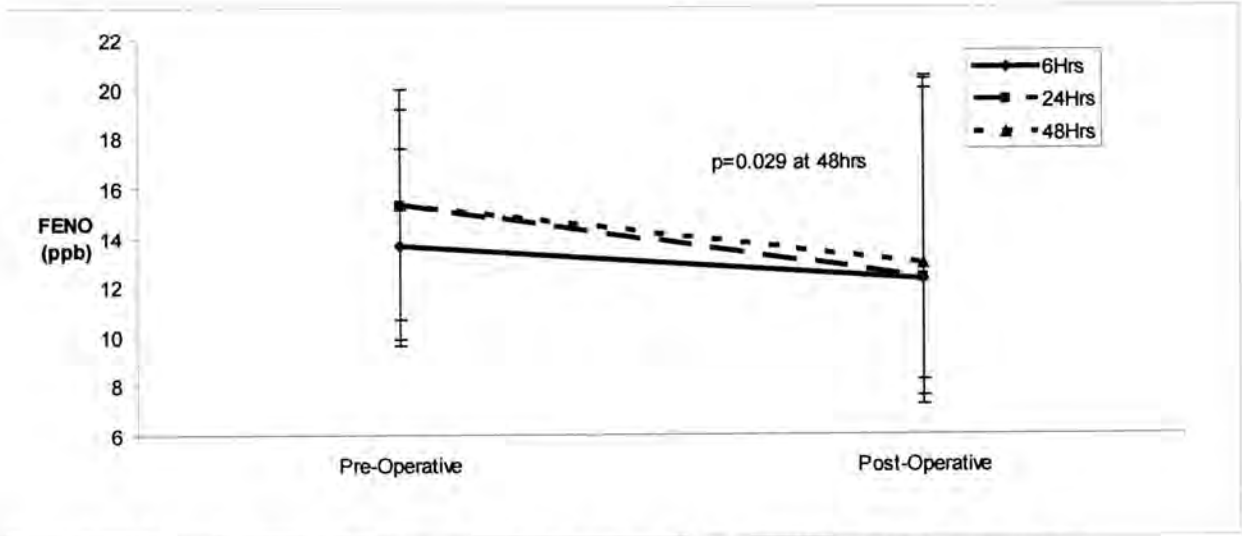


Figure 3.20 Post-Operative Change in Exhaled Nitric Oxide (FENO). The data were not normally distributed thus median exhaled nitric oxide measurements from the Niox Flex are shown. Error bars represent the interquartile range. The pre-operative sample was compared to the three post-operative time points using a paired t-test. Due to some patients not being able to complete all three post-operative measurements, the pairing to post-operative results led to slightly different pre-operative median values for each paired t-test (pre-operative n=40, 6hrs post-operative n=31, 24 and 48 hrs post-operative n=32). There was a general trend towards reduction in exhaled nitric oxide which reached significance at 48 hrs (geometric mean ratio 1.2 p=0.029). ANOVA modelling found no significant post-operative change for FENO.

Variable flow rates (30mls/hr, 50mls/hr and 100mls/hr) enabled the alveolar nitric oxide to be calculated at each time point using the two-compartment model of airway dynamics. However, there was no significant change found (Figure 3.21). ANOVA modelling did not reveal a significant post-operative change in FENO or alveolar nitric oxide or difference between surgical approaches.

Pre-operative FENO measurements and alveolar nitric oxide calculations from patients were compared with the control group of a similar age (Table 3.13). No statistically significant differences were found although there was a notably higher median value of FENO in the control group which fell short of significance (geometric mean ratio 1.49 95% CI 0.99-2.25 p=0.055).

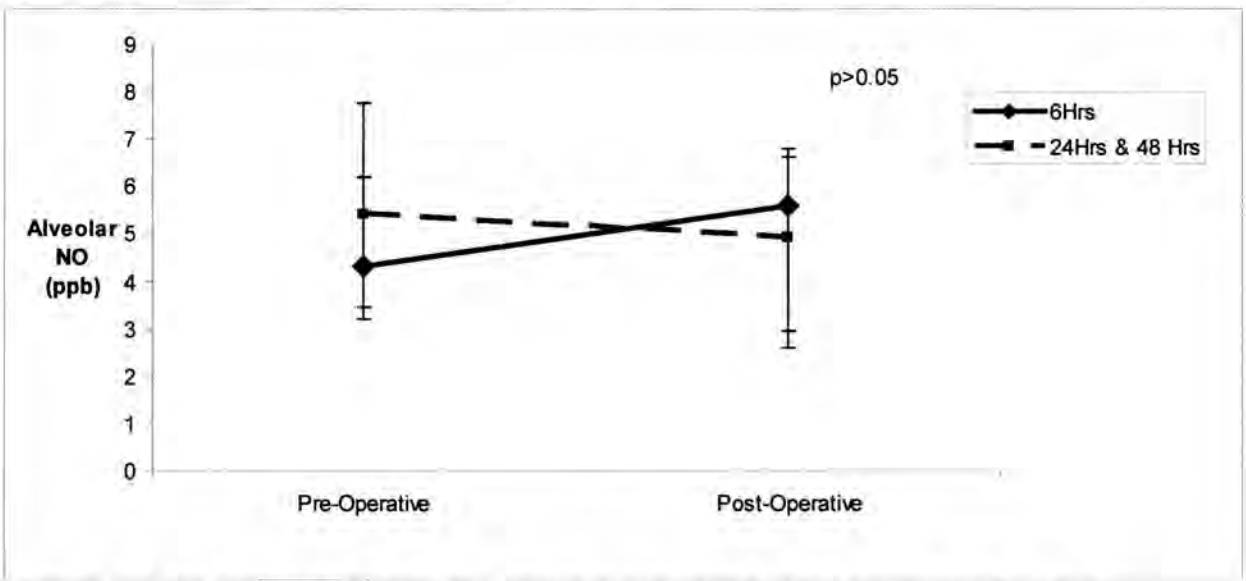


Figure 3.21 Post-Operative Change in Derived Alveolar Nitric Oxide (FENO). The data were not normally distributed thus median values for alveolar nitric oxide are shown. Error bars represent the interquartile ranges. The measurements were derived using the two-compartment model of airway dynamics and variable flow rates of 30ml/second, 50mls/second and 100mls/second. The pre-operative sample was compared to the three post-operative time points using a Paired t-test after $\log_{10}$ transformation. Due to some patients not being able to complete all three post-operative measurements at three flow rates, the pairing to post-operative results led to slightly different pre-operative median values for each paired t-test (pre-operative n=39, 6hrs post-operative n=29, 24hrs n=27 and 48 hrs post-operative n=30). No statistically significant difference was found post-operatively at any time point and ANOVA modelling found no significant post-operative change for alveolar nitric oxide.

	Pre-Operative Patient Median and (IQR) (ppb)	Control Patient Median and (IQR) (ppb)	p-value
FENO	14.1 (8.8-18.3)	23.3 (21.0-30.7)	0.055
Derived Alveolar NO	4.9 (3.5-6.5)	5.6 (3.9-7.7)	0.913

Table 3.13 Comparison of Patient and Control Group Exhaled Nitric Oxide. The data were not normally distributed thus median and interquartile (IQR) ranges are shown. The data was $\log_{10}$ transformed prior to using a two-sample t-test. No statistically significant differences were found ($p<0.05$). Measurements from the Niox Flex are shown. n=40 for patient FENO, n=39 for patient derived alveolar NO and n=10 for controls.

IV - RESULTS –Biomarkers of Post-Operative Pneumonia Following Major Anatomic Lung Cancer Resection for Clinically Suspected Lung Cancer

The principal purpose of the study was to examine innate immune parameters peri-operatively in patients undergoing lung cancer resection to identify any significant differences between those patients whom subsequently developed post-operative pneumonia and those whom did not with a view to identifying early biomarkers. The primary hypothesis proposed that patients have either heightened or suppressed immune function pre-operatively or early in the post-operative course which could predispose them to a hospital acquired pneumonia.

Prospective criteria for diagnosis of post-operative pneumonia were defined. The essential component was consolidation on the 72-hour post-operative chest roentogram, blindly reported by a Consultant Radiologist, plus at least two of WBC count >11.0 or <4.0x10^9/l, temperature >38.0°C or <35.5°C or positive microbiology culture from sputum. Patients were divided into pneumonia or no pneumonia groups and innate immune parameters compared. Significantly different parameters were subsequently evaluated for their individual predictive strength for post-operative pneumonia.

Patient Background

Forty patients enrolled in the study underwent lung cancer resection. Fourteen patients (35%) met the prospectively defined criteria for post-operative pneumonia. No patient fulfilled the prospectively defined diagnostic criteria for ALI/ARDS (Chapter II, Clinical Definitions).

Table 4.1 compares the clinical characteristics of the two groups. There were no statistically significant differences found except for T_{co} (60.9% predicted cf. 77.9% predicted p=0.010). Other measures of lung function were lower in pneumonia patients but these were not significantly different to other patients. Tables 4.2 and 4.3 summarise

the final histopathology and the TNM lung cancer stage respectively of the groups. There were no statistically significant differences found. Although the study targeted lung cancer patients, two patients in the no pneumonia group were discovered to have a diagnosis other than lung cancer post-operatively (metastatic colorectal carcinoma and Wegener's granulomatosis).

The specific operations performed in each group are detailed in Table 4.4. It is important to note that approximately two-thirds of all cases were undertaken using VATS surgery explaining why approximately two-thirds of pneumonia cases were VATS cases. No statistically significant difference in terms of operation performed was found between the two groups.

Table 4.5 compares the two groups in terms of other post-operative complications. There were no significant differences between the groups for individual complications although pneumonia patients were more likely to suffer another major complication including respiratory failure, retention of secretions requiring mini-tracheostomy and/or suction bronchoscopy, stroke, bowel obstruction and acute renal failure.

Characteristic	All Patients n=40	Pneumonia n=14	No Pneumonia n=26	p-value
Male, n (%)	23 (57.5)	8 (57.1)	15 (57.7)	0.973
Mean Age, years (SD)	66.9 (7.8)	67.5 (9.4)	66.2 (7.1)	0.645
Mean BMI, kg/m^2 (SD)	26.6 (4.3)	26.7 (4.1)	26.6 (4.4)	0.949
Current Smoker, n (%)*	13 (32.5)	4 (28.6)	9 (34.6)	0.692
Mean FEV_1, % Predicted (SD)	87.3 (23.8)	83.3 (21.0)	91.3 (25.0)	0.291
Mean FEV_1/FVC, % Predicted (SD)	88.5 (13.9)	89.7 (14.6)	87.3 (13.8)	0.611
Mean T_{CO}, % Predicted (SD)	72.0 (21.9)	60.9 (16.3)	77.9 (22.4)	**0.010**
Mean K_{CO}, % Predicted (SD)	78.1 (17.6)	72.4 (17.7)	83.7 (16.5)	0.060
Mean Thoracoscore, % (SD)	2.5 (1.7)	2.95 (2.3)	2.04 (1.2)	0.190
Inpatient Mortality, n (%)	0 (0)	0 (0)	0 (0)	1.000
Geometric Mean Blood Loss (SD)	139 (2.7)	155 (2.7)	122 (2.7)	0.482
Mean Change in Blood Hb g/l (Pre-op-Day 1), n (SD)	13.8 (10.0)	16.5 (8.9)	11.1 (10.2)	0.095
Mean Duration of Surgery, Minutes (SD)	203 (44.4)	207 (44.4)	198 (44.3)	0.566
Geometric Mean Post-Operative Stay (SD)	6.3 (1.7)	7.0 (2.1)	5.6 (1.5)	0.333

*Current or ex-smoker <2 months duration

Table 4.1 Key Clinical Characteristics of Patients Developing Post-Operative Pneumonia Compared with No Pneumonia. Data are presented as means (and standard deviation) or n (%) as appropriate. P values refer to the comparison of pneumonia versus no-pneumonia groups, with statistical analysis using an unpaired t-test for continuous variables or the two proportion test for non-continuous variables (or Fisher's exact test where numbers were small). Data that were not normally distributed (as determined by the Anderson-Darling test) were log_{10} transformed prior to further analysis and reported as a geometric mean. Prior to transformation blood loss was reported in millilitres (mls) and post-operative stay in days. BMI, body mass index; FEV_1, forced expiratory volume in 1 second; FVC, forced vital capacity; Thoracoscore is risk stratification system for inpatient mortality using a number of pre-operative patient characteristics (Falcoz et al., 1997); Hb, haemoglobin; Tco, transfer factor for carbon monoxide; Kco, transfer factor for carbon monoxide, corrected for lung volume.

Final Histopathology Diagnosis	All Patients n=40	Pneumonia n=14	No Pneumonia n=26	p-value
Adenocarcinoma, n (%)	17 (42.5)	8 (57.1)	9 (34.6)	0.164
Squamous Cell Carcinoma, n (%)	14 (35.0)	3 (21.4)	11 (42.3)	0.154
Bronchoalveolar Carcinoma, n (%)	3 (7.5)	1 (7.1)	2 (7.7)	1.000
Large Cell Carcinoma, n (%)	1 (2.5)	1 (7.1)	0 (0)	0.350
Pleomorphic Carcinoma, n (%)	1 (2.5)	0 (0)	1 (3.8)	1.000
Synchronous Pleomorphic Carcinoma & Adenocarcinoma, n (%)	1 (2.5)	1 (7.1)	0 (0)	0.350
Atypical Carcinoid, n (%)	1 (2.5)	0 (0)	1 (3.8)	1.000
Metastatic Colorectal Adenocarcinoma, n (%)	1 (2.5)	0 (0)	1 (3.8)	1.000
Wegener's Granulomatosis, n (%)	1 (2.5)	0 (0)	1 (3.8)	1.000

Table 4.2 Final Histopathological Diagnosis from Resected Lung Specimens. P-values refer to the comparison between pneumonia and no pneumonia groups derived using the two proportion test (or Fisher's exact test where numbers were small). No statistically significant differences were found.

Lung Cancer TNM Stage	All Patients n=38 n (%)	Pneumonia n=14 n (%)	No Pneumonia n=24 n (%)	p-value
Total Stage IA	**13 (34.2)**	**6 (42.9)**	**7 (29.2)**	0.397
T1a N0 M0	6 (15.8)	2 (14.3)	4 (16.7)	
T1b N0 M0	7 (18.4)	4 (28.6)	3 (12.5)	
Total Stage IB	**8 (21.1)**	**2 (14.3)**	**6 (25.0)**	0.684
T2a N0 M0	8 (21.1)	2 (14.3)	6 (24.0)	
Total Stage IIA	**6 (15.8)**	**3 (21.4)**	**3 (12.5)**	0.650
T2b N0 M0	1 (2.6)	0 (0)	1 (4.2)	
T1a N1 M0	1 (2.6)	1 (7.14)	0 (0)	
T1b N1 M0	1 (2.6)	1 (7.14)	0 (0)	
T2a N1 M0	3 (7.9)	1 (7.14)	2 (8.3)	
Total Stage IIB	**2 (5.2)**	**1 (7.14)**	**1 (4.2)**	1.000
T2b N1 M0	1 (2.6)	0 (0)	1 (4.2)	
T3 N0 MX	1 (2.6)	1 (7.14)	0 (0)	
Total Stage IIIA	**8 (21.1)**	**1 (7.14)**	**7 (29.2)**	0.216
T2b N2/N3 M0	1 (2.6)	0 (0)	1 (4.2)	
T3 N1 M0	2 (5.3)	1 (7.14)	1 (4.2)	
T3 N2 M0	2 (5.3)	0 (0)	2 (8.3)	
T4 N0 M0	3 (7.9)	0 (0)	3 (12.5)	
Total Stage IIIB	**1 (2.6)**	**1 (7.14)**	**0 (0)**	0.368
T4 N2 M0	1 (2.6)	1 (7.14)	0 (0)	

Table 4.3 Final Pathological Lung Cancer TNM Stage. P-values refer to pneumonia versus no pneumonia groups derived using the two-proportion t-test (or Fisher's exact test where numbers were small). No statistically significant differences were found.

Operation	All Patients n=40 n (%)	Pneumonia n=14 n (%)	No Pneumonia n=26 n (%)	p-value
VATS Lung Resection	**26 (65.0)**	**9 (64.3)**	**17 (65.4)**	0.945
VATS Left Upper Lobectomy	9 (22.5)	2 (14.3)	7 (26.9)	0.322
VATS Left Lower Lobectomy	1 (2.5)	1 (7.1)	0 (0)	0.350
VATS Right Upper Lobectomy	10 (25.0)	4 (28.6)	6 (23.1)	0.707
VATS Right Middle Lobectomy	3 (7.5)	2 (14.3)	1 (3.8)	0.276
VATS Right Lower Lobectomy	1 (2.5)	0 (0)	1 (3.8)	1.000
VATS Right Middle and Lower Lobectomy	1 (2.5)	0 (0)	1 (3.8)	1.000
VATS Right Basal Segmentectomy	1 (2.5)	0 (0)	1 (3.8)	1.000
Lung Resection via Thoracotomy	**14 (35.0)**	**5 (35.7)**	**9 (34.6)**	0.945
Left Upper Lobectomy	5 (12.5)	3 (21.4)	2 (7.7)	0.258
Right Middle Lobectomy	1 (2.5)	1 (7.1)	0 (0)	0.350
Right Lower Lobectomy	1 (2.5)	0 (0)	1 (3.8)	1.000
Left Upper Lobectomy with Vascular Sleeve	1 (2.5)	0 (0)	1 (3.8)	1.000
Left Upper Lobectomy with Vascular and Bronchial Sleeve	1 (2.5)	0 (0)	1 (3.8)	1.000
Left Lower Lobectomy, Wedge Excision of Left Upper Lobe	1 (2.5)	0 (0)	1 (3.8)	1.000
Right Upper Lobectomy, Wedge of Middle Lobe & Chest Wall Resection	1 (2.5)	1 (7.1)	0 (0)	0.350
Right Middle and Lower Lobectomy	1 (2.5)	0 (0)	1 (3.8)	1.000
Left Pneumonectomy	1 (2.5)	0 (0)	1 (3.8)	1.000
Right Pneumonectomy	1 (2.5)	0 (0)	1 (3.8)	1.000

Table 4.4 Lung Resection Performed and Incidence of Post-Operative Pneumonia. All operations were combined with an adenectomy. P-values were calculated using the two-proportion t-test or where numbers were small the Fisher's exact test. There were no statistically significant differences found.

Complication	All Patients n=40 n (%)	Pneumonia n=14 n (%)	No Pneumonia n=26 n (%)	p-value
In-patient Mortality	0 (0.0)	0 (0)	0 (0)	1.000
Respiratory Failure requiring Re-intubation	1 (2.5)	1 (7.1)	0 (0)	0.350
Mini-Tracheostomy and/or Suction Bronchoscopy	2 (5.0)	2 (14.3)	0 (0)	0.117
Bleeding requiring Surgical Re-exploration	1 (2.5)	1 (7.1)	0 (0.0)	0.350
Stroke	1 (2.5)	1 (7.1)	0 (0.0)	0.350
Bowel Obstruction	1 (2.5)	0 (0.0)	1 (3.8)	1.000
Acute Renal Failure	1 (2.5)	1 (7.1)	0 (0.0)	0.350
Air Leak >5 days	7 (17.5)	3 (21.4)	4 (15.4)	0.679
Atrial Fibrillation	5 (12.5)	3 (21.4)	2 (7.7)	0.322
Pericarditis	1 (2.5)	1 (7.1)	0 (0.0)	0.350
Temporary Heart Block (2nd Degree)	1 (2.5)	1 (7.1)	0 (0.0)	0.350
Clostridium difficile Diarrhoea	1 (2.5)	0 (0.0)	1 (3.8)	1.000
Acute Urinary Retention	1 (2.5)	0 (0.0)	1 (3.8)	1.000
Urinary Tract Infection	1 (2.5)	0 (0.0)	1 (3.8)	1.000
Wound Cellulitis	1 (2.5)	1 (7.1)	0 (0.0)	0.350
Excessive Pain	3 (7.5)	1 (7.1)	2 (7.7)	1.000
Opiate Toxicity/Confusion/Agitation	3 (7.5)	2 (14.3)	1 (3.8)	0.276
Nausea and Vomiting	1 (2.5)	1 (7.1)	0 (0.0)	0.350

Table 4.5 Comparison of Other Post-Operative Complications Between Pneumonia and No Pneumonia Patients. P-values refer to pneumonia versus no pneumonia groups and were calculated using the Fisher's exact test. No statistically significant differences were found.

Serum Cytokines: IL-8, TNF-α, IL-12, IL-10, IL-1β and IL-6

Six serum cytokines were compared between pneumonia and no pneumonia patients. There were no statistically significant differences found between the groups pre-operatively but post-operatively significant differences emerged for IL-8 and TNF-α. Figure 4.1 shows the group trends for IL-8. Pneumonia patients had higher levels of IL-8 at each time point which peaked at 24hrs. The difference in post-operative change between the groups was statistically significant at 48hrs (geometric mean ratio 1.43 95% CI 1.04-1.97 p=0.031). ANOVA modelling found no overall significant difference for IL-8 between pneumonia and no pneumonia groups (p=0.109).

Figure 4.2 demonstrates the post-operative change in TNF-α for pneumonia compared with no pneumonia patients. It is important to note that there was no significant change in TNF-α post-operatively for all patients (please refer to serum cytokines in chapter III). However, a divergence in responses was found between the groups with an initial fall in TNF-α for pneumonia patients and an increase in no pneumonia patients at 6hrs post-operatively. The small difference between the groups reached statistical significance at 24hrs (mean difference 2.259pg/ml p=0.023). By 48hrs both groups had increased TNF-α levels. ANOVA modelling however found no statistically significant difference post-operatively between the groups (p=0.729). No statistically significant differences were found for the other measured cytokines. The post-operative trends between the two groups are shown in Figure 4.3.

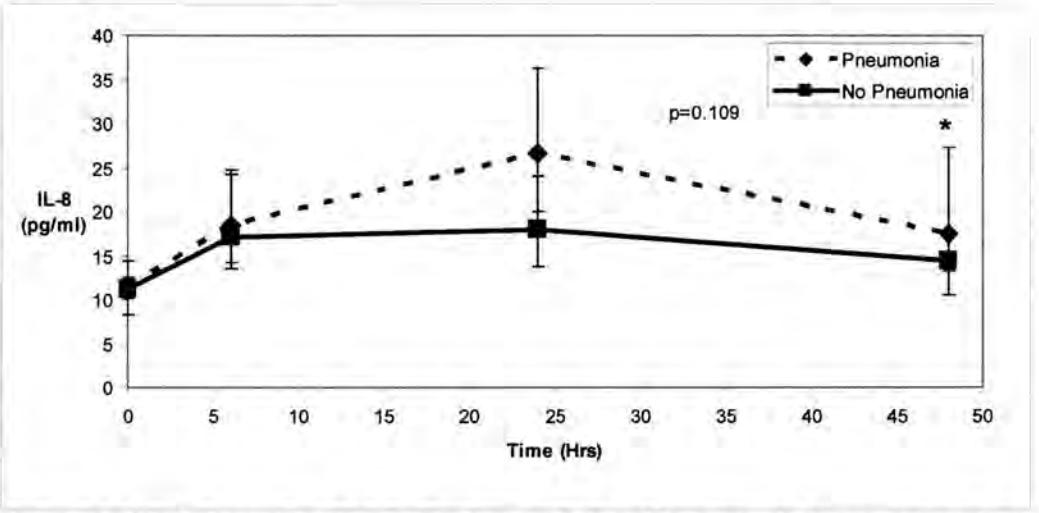


Figure 4.1 Post-Operative Change in Serum IL-8 for Pneumonia Compared with No Pneumonia Patients. The data were not normally distributed thus median values of IL-8 are shown. Error bars represent the interquartile range. The post-operative change was calculated and $\log_{10}$ transformed. Pneumonia versus no pneumonia groups were compared using a two sample t-test. Pneumonia patients had a larger change in IL-8 post-operatively which was significant at 48hrs (highlighted by the * symbol, geometric mean ratio 1.43 p=0.031). However, ANOVA modelling did not reveal an overall difference in IL-8 for pneumonia versus no pneumonia patients (p-value on chart). N=40 at each time point except at 48hrs post-operatively where n=38.

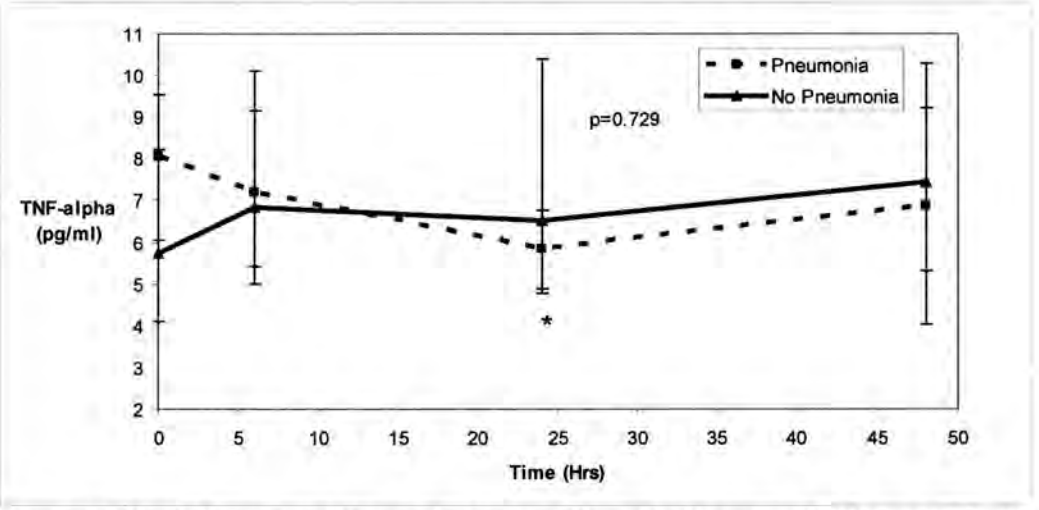
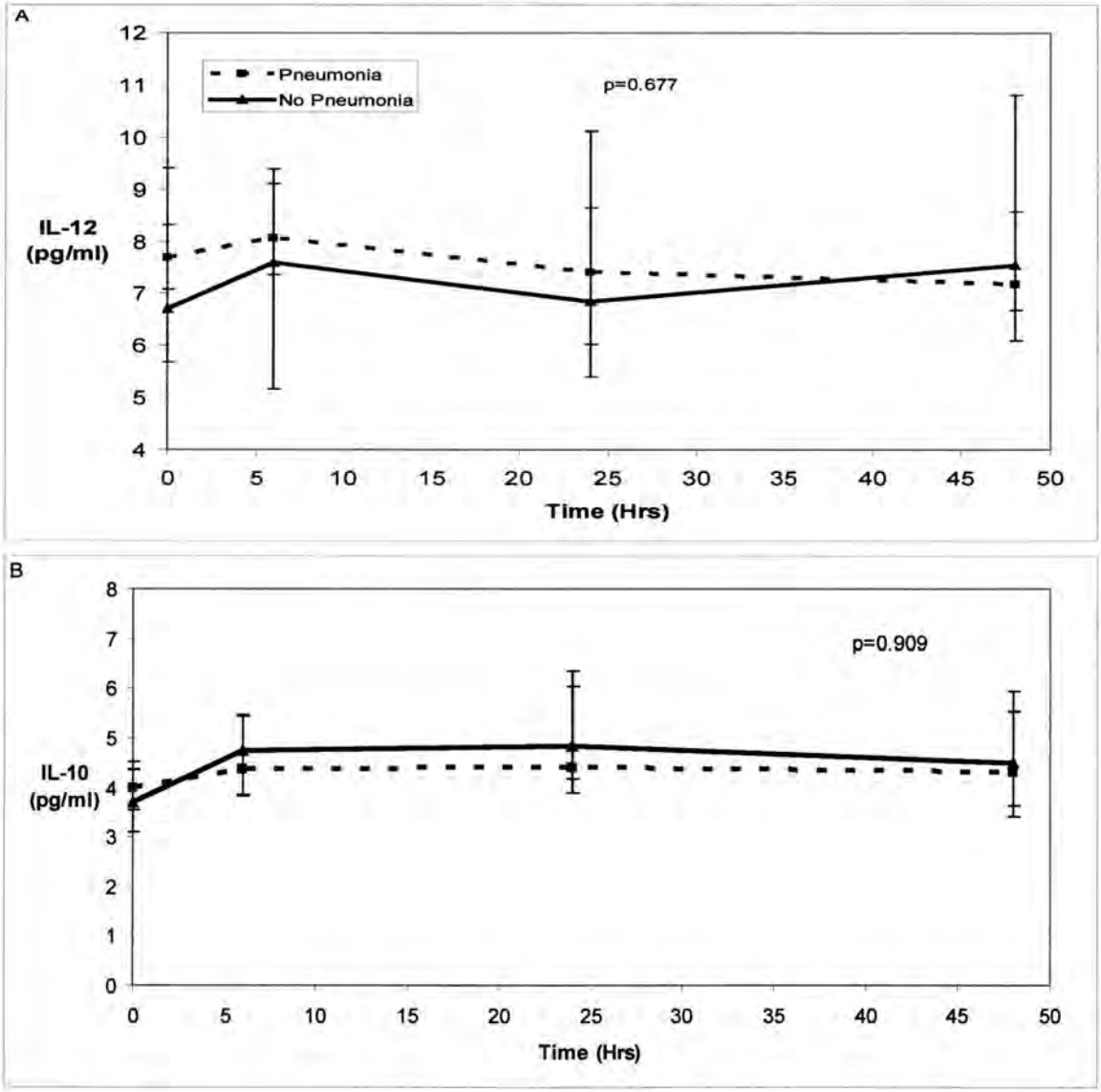
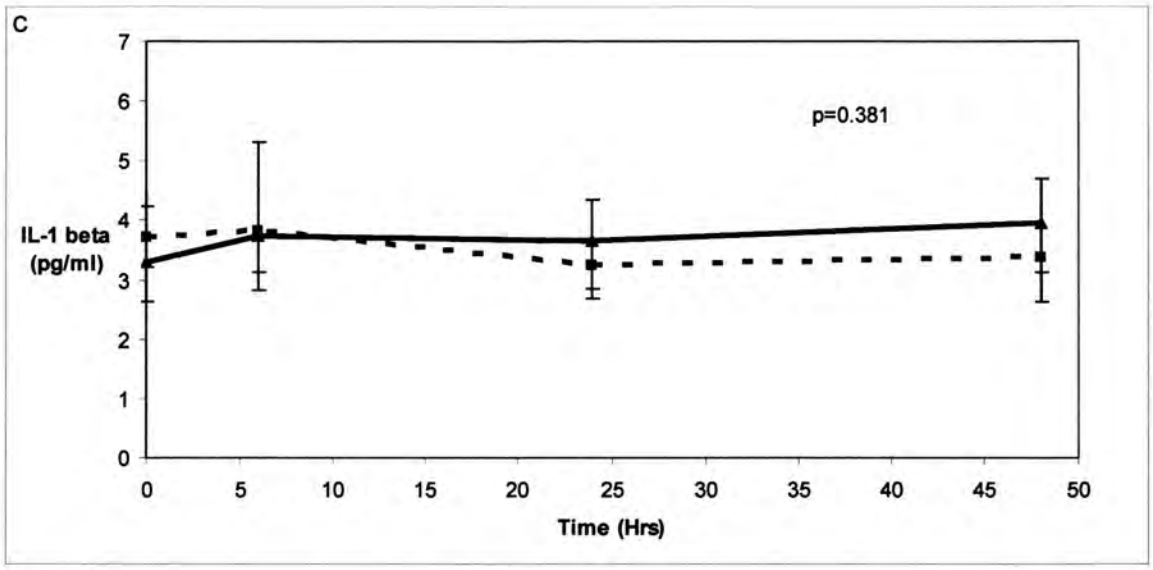
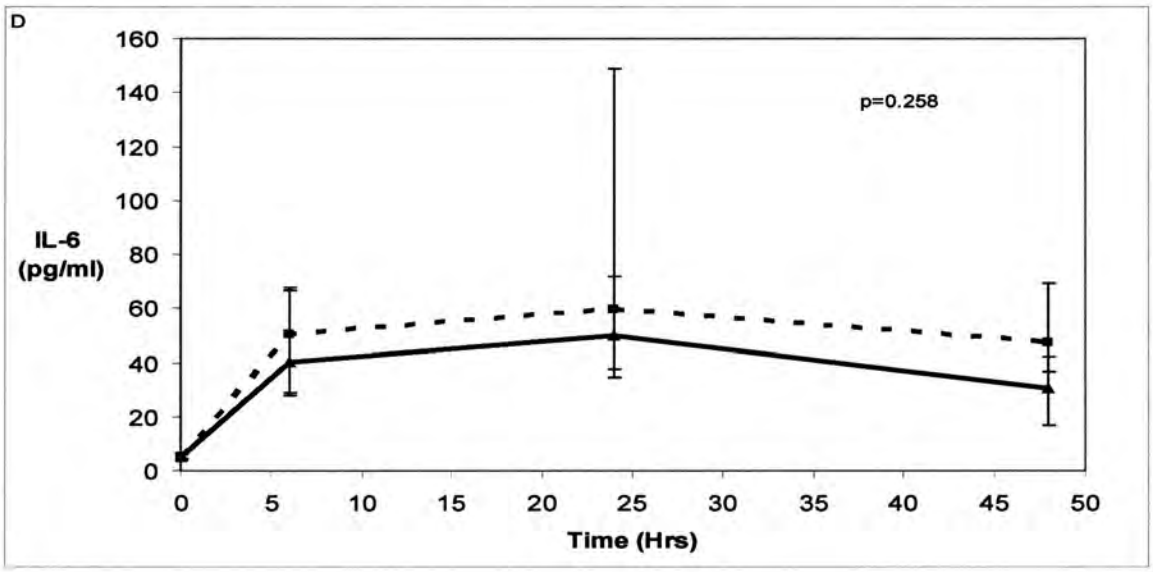


Figure 4.2 Post-Operative Change in Serum TNF-α for Pneumonia Compared with No Pneumonia Patients. The data were not normally distributed thus median values of TNF-α were calculated. Error bars represent the interquartile range. The post-operative change was calculated and $\log_{10}$ transformed. Pneumonia versus non-pneumonia groups were compared using a two sample t-test. Although there was no significant overall post-operative change, there was a statistically significant difference between the groups at 24hrs (highlighted by the * symbol, mean difference 2.259pg/ml p=0.023). However, ANOVA modelling did not reveal an overall difference in TNF-α for pneumonia versus no pneumonia patients (p value on chart). N=40 at each time point except 48hrs post-operatively where n=38.

Figure 4.3 Post-Operative Changes in Serum IL-12, IL-10, IL-1β and IL-6 for Pneumonia versus No Pneumonia Patients. [Panels A-D] The data were not normally distributed thus median values are shown below. Error bars represent the interquartile range. The post-operative change was calculated and $\log_{10}$ transformed. Pneumonia versus non-pneumonia groups were compared using a two-sample t-test. No statistically significant difference was found at any time point or on ANOVA modelling (ANOVA p value shown on charts).




C
IL-1 beta (pg/ml)
p=0.381
Time (Hrs)


D
IL-6 (pg/ml)
p=0.258
Time (Hrs)

Leucocyte, Neutrophil, Monocyte, Platelet and Serum Albumin Counts

Routine NHS haematology and biochemistry counts were analysed to determine if any early changes emerged for pneumonia compared with no pneumonia patients. Figure 4.4 compares the pre-operative leucocyte and neutrophil count between the groups. Patients subsequently developing pneumonia had higher pre-operative leucocyte counts (geometric mean ratio 1.26 95% CI 1.07-1.49 p=0.007) and higher neutrophil counts (geometric mean ratio 1.31 95% CI 1.06-1.63 p=0.015) compared with no pneumonia patients. The values however were within the normal NHS reporting range. There was no statistically significant difference in pre-operative monocyte or lymphocyte count between the groups.

The post-operative trend in leucocyte count between the groups is profiled in Figure 4.5. ANOVA modelling revealed a significant overall post-operative difference between pneumonia and no pneumonia groups (p=0.017) despite individual time point t-tests showing no significant difference between the groups.

The post-operative trend in neutrophil count mirrored the leucocyte count (Figure 4.6). ANOVA modelling revealed an overall significant difference between pneumonia and non-pneumonia patients (p=0.024). Two sample t-tests for individual time points revealed no difference between the groups.

There was no significant difference for change in monocyte count between pneumonia and no pneumonia patients (data not shown). However, ANOVA modelling identified a complex interaction between time, pneumonia or not and VATS versus open surgery (p=0.005). The numbers were too small however for meaningful comparisons to be drawn.

Figure 4.7 summarises the post-operative trend in serum albumin between the two groups. ANOVA modelling identified significantly lower levels of serum albumin in

pneumonia patients compared with no pneumonia patients (p=0.020) although there was no significant difference found at any specific time point. A complex interaction was identified between time point, type of surgery and pneumonia or not although the numbers were too small to allow meaningful sub-group analysis. No significant difference was identified between the groups for baseline platelet count or post-operative change in platelets (data not shown).

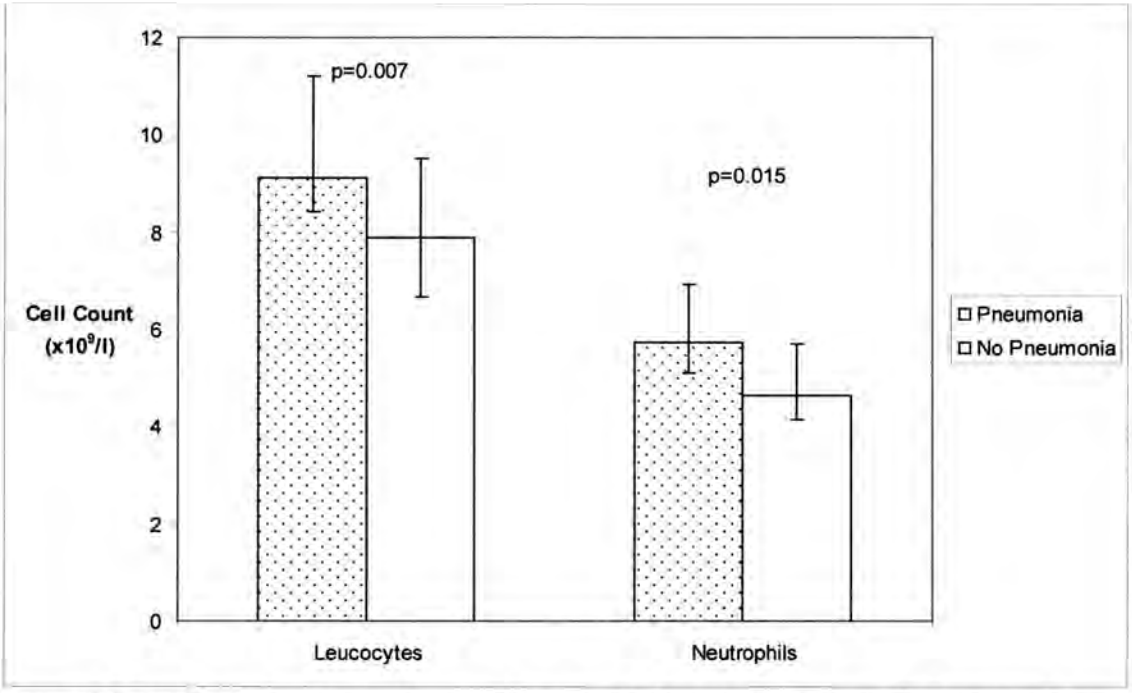


Figure 4.4 Pre-Operative Leucocyte and Neutrophil Counts in Patients Subsequently Developing a Post-Operative Pneumonia. The data were not normally distributed thus median values are shown. Error bars represent the interquartile ranges. The data was $\log_{10}$ transformed prior to performing a two sample t-test. Patients whom developed post-operative pneumonia had a relatively high leucocyte count pre-operatively (geometric mean ratio 1.26 p=0.007). This was due to a relatively high pre-operative neutrophil count (geometric mean ratio 1.3 p=0.015). The data were derived from NHS Lothian Haematology Laboratory. n=40.

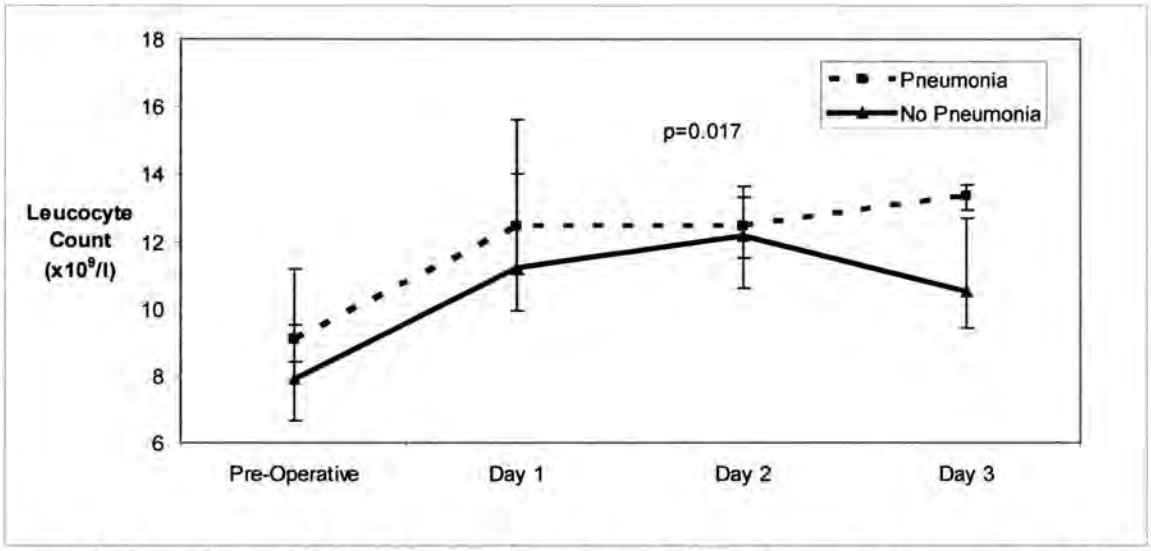


Figure 4.5 Post-Operative Trend in Leucocyte Count Differs Between Pneumonia and No Pneumonia Patients. The data were not normally distributed thus the median value at each time point is shown. Error bars represent the interquartile range. Post-operative change was calculated and $\log_{10}$ transformed prior to further analysis. A two-sample t-test did not reveal any statistically significant differences between the groups. However, ANOVA modelling revealed an overall significant difference between pneumonia and non-pneumonia patients (p value on chart). Data were derived from NHS Lothian Haematology Laboratory. n=40 pre-operatively and D1, n=30 D2 and n=26 D3.

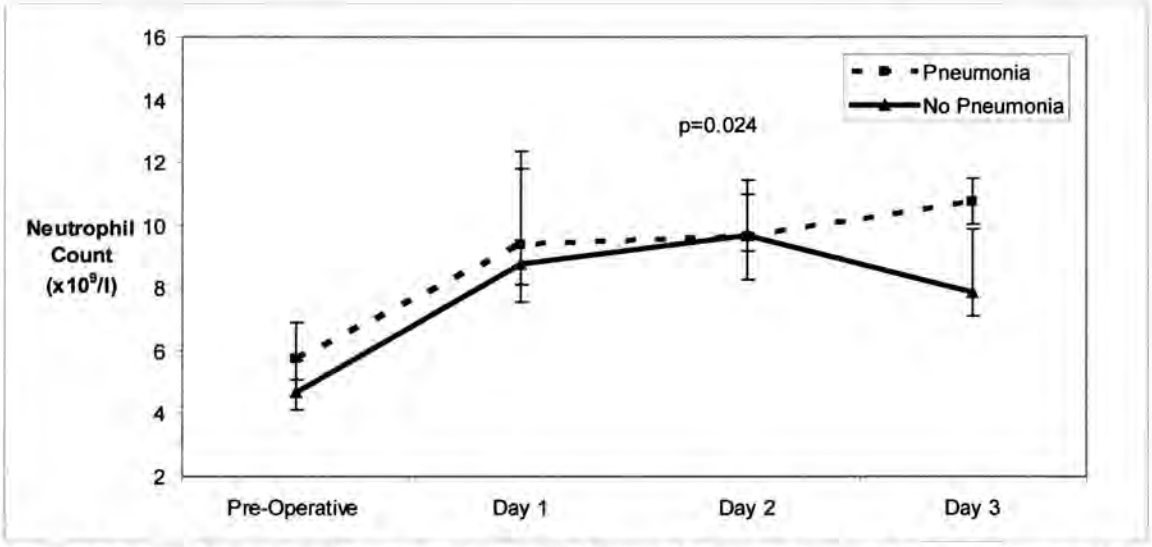


Figure 4.6 Post-Operative Trend in Neutrophil Count Differs Between Pneumonia and No Pneumonia Patients. The data were not normally distributed thus the median value at each time point is shown. Error bars represent the interquartile range. Post-operative change was calculated and $\log_{10}$ transformation prior to further analysis. A two-sample t-test did not reveal any statistically significant differences between the groups. However, ANOVA modelling revealed an overall significant difference between pneumonia and non-pneumonia patients (p value on chart). Data were derived from NHS Lothian Haematology Laboratory n=40 pre-operatively and D1, n=30 D2 and n=26 D3.

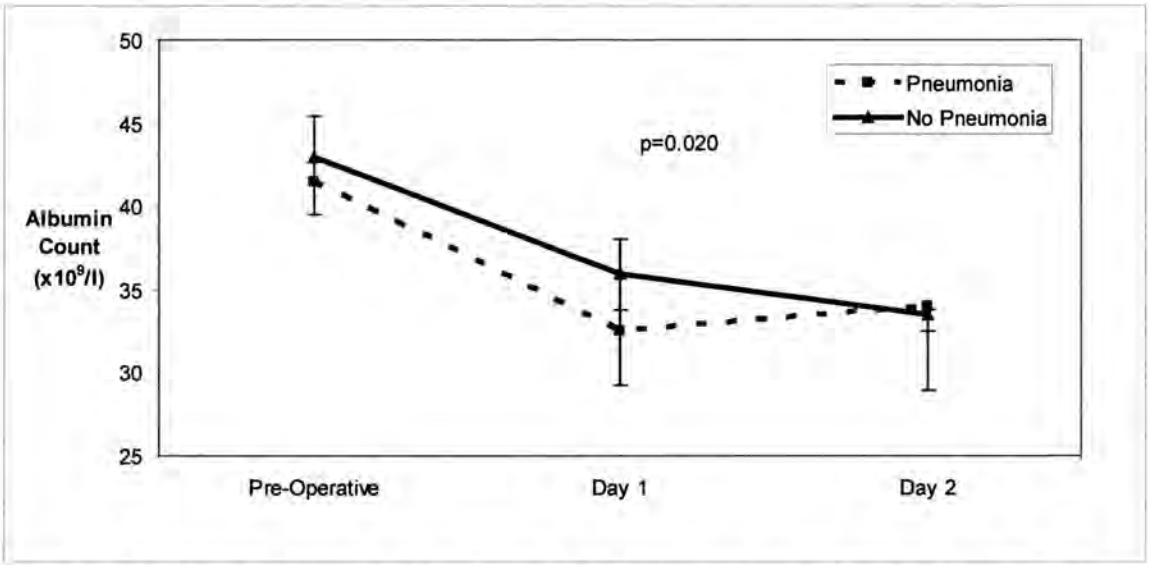
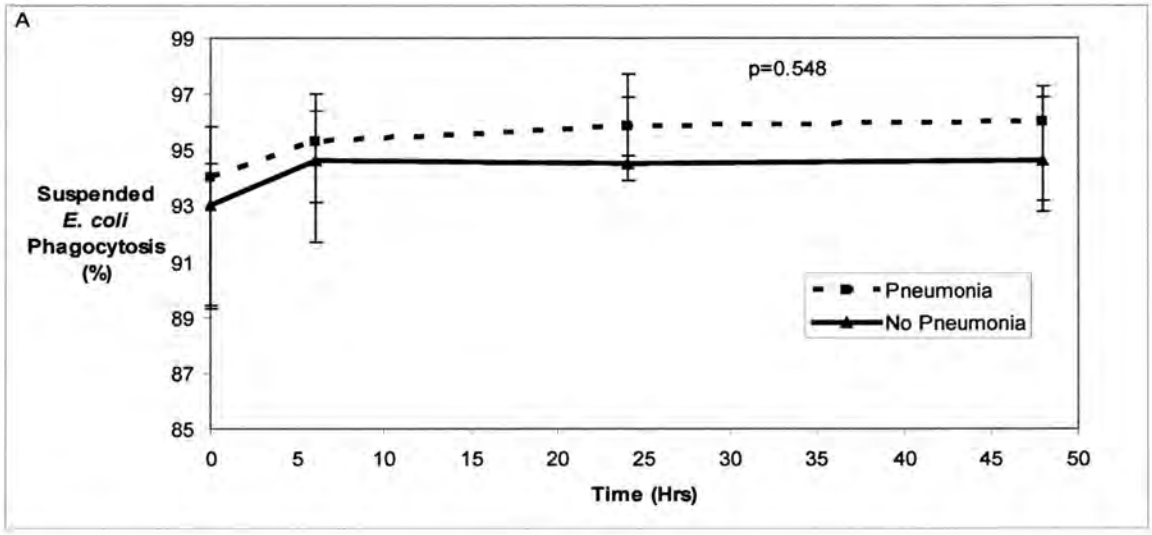


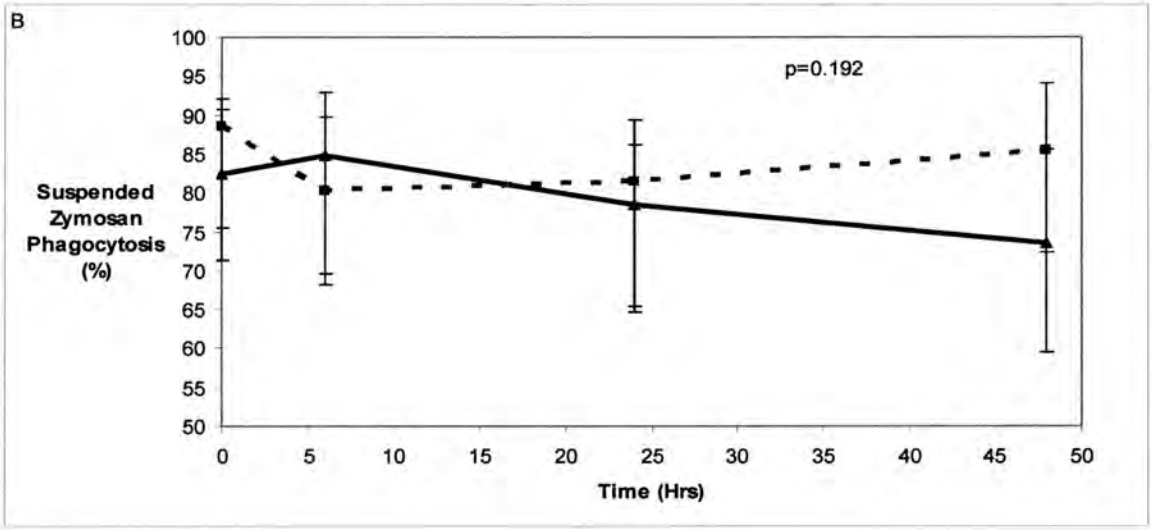
Figure 4.7 Pneumonia Patients Have Lower Early Post-Operative Albumin Counts. The median value at each time point is shown as the data was not normally distributed. Error bars represent the interquartile range. Post-operative change was calculated and the data $\log_{10}$ transformed prior to further analysis. A two-sample t-test did not reveal any statistically significant differences between the groups. ANOVA modelling however showed a significant overall difference between pneumonia and no pneumonia (p value on chart) and type of surgery (p=0.001). n=40 pre-operatively, n=35 D1 and n=21 D2.

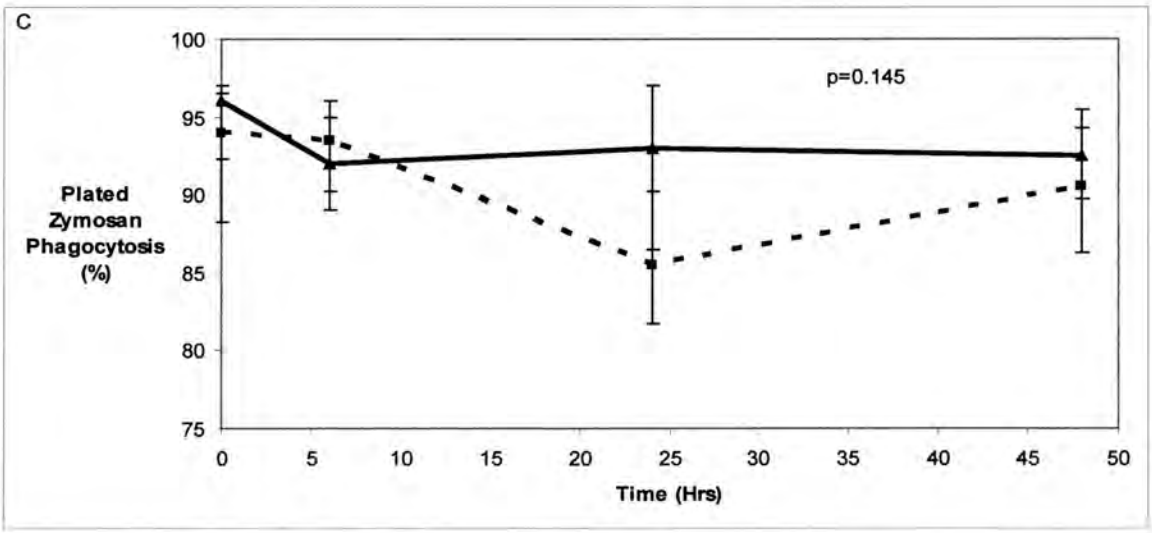
Neutrophil Phagocytosis

The data above identified higher leucocyte and neutrophil counts pre-operatively in pneumonia patients compared with no pneumonia patients with an overall higher post-operative trend on ANOVA modelling. The functional capacity of neutrophils was also assessed peri-operatively using three assays of phagocytosis. Figure 4.8 summarises the trends of pneumonia and no pneumonia groups. No significant differences were found pre-operatively or post-operatively.

Figure 4.8 Summary of Post-Operative Trends in Neutrophil Phagocytosis for Pneumonia and No Pneumonia Patients. [Panels A-C] The data were not normally distributed thus the median phagocytosis percentage is shown. Error bars represent the interquartile range. The data was $\log_{10}$ transformed and the groups compared with a two sample t-test which revealed no significant difference at any time point between the groups. ANOVA modelling confirmed no overall significant difference between pneumonia and no pneumonia groups (p values on charts). n=37 for suspended *E.coli* phagocytosis, n=38 for suspended zymosan phagocytosis and n=40 for plated phagocytosis except n=38 at 48hrs.


A
p=0.548
Suspended E. coli Phagocytosis (%)
99
97
95
93
91
89
87
85
0
5
10
15
20
25
30
35
40
45
50
Time (Hrs)
Pneumonia
No Pneumonia


B
p=0.192
Suspended Zymosan Phagocytosis (%)
100
95
90
85
80
75
70
65
60
55
50
0
5
10
15
20
25
30
35
40
45
50
Time (Hrs)


C
p=0.145
Plated Zymosan Phagocytosis (%)
100
95
90
85
80
75
0
5
10
15
20
25
30
35
40
45
50
Time (Hrs)

BAL

BAL was performed pre-operatively at the time of the staging bronchoscopy prior to mediastinoscopy and immediately after lung resection. Each lavage was undertaken from a different lobe of the contralateral lung to the culprit lesion. This provided a unique insight into the early inflammatory response to surgery in the non-operated lung and any early differences to be identified between those patients subsequently developing post-operative pneumonia and those not.

The overall concentration of cells in BAL sampled pre-operatively was found to be higher in patients subsequently developing post-operative pneumonia (geometric mean ratio between the groups was 2.07 95% CI 1.19-3.6 p=0.012, Figure 4.9). No significant difference was found between the groups for the change in BAL cellular concentration post-operatively. The median neutrophil count was slightly higher in pneumonia patients post-operatively although this was not statistically significant (Table 4.6).

BAL cytokines (IL-1β, IL-6, IL-8, IL-10, IL-12 and TNF-α) were compared between the two groups at baseline and post-operatively. No statistically significant differences were found (ANCOVA p-values were IL-12 p=0.472, TNF-α p=0.488, IL-10 p=0.200, IL-6 p=0.251, IL-1β p=0.760 and IL-8 p=0.780). Figure 4.10 details the trends for each cytokine in both groups.

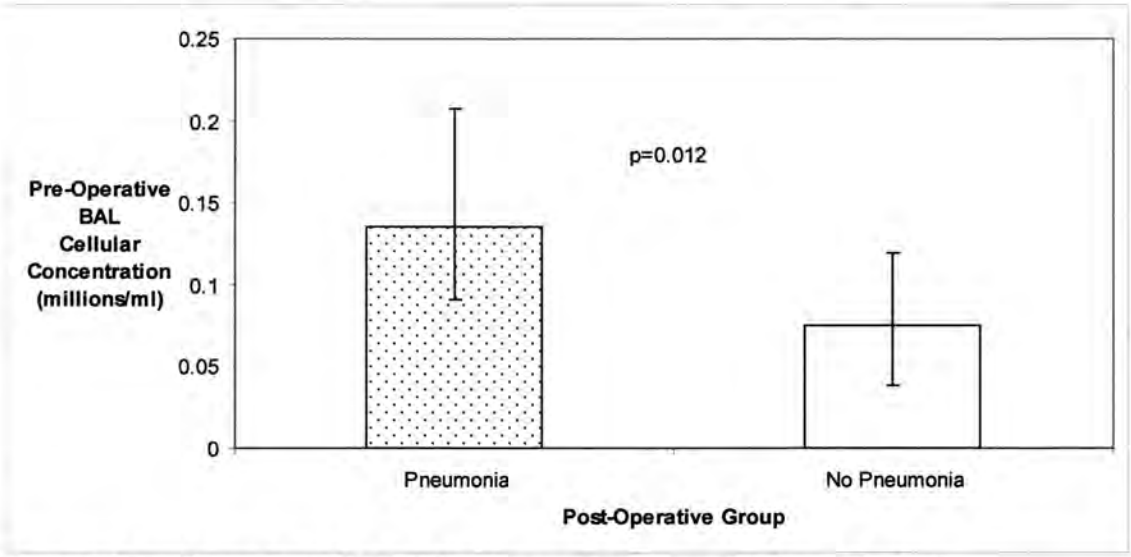
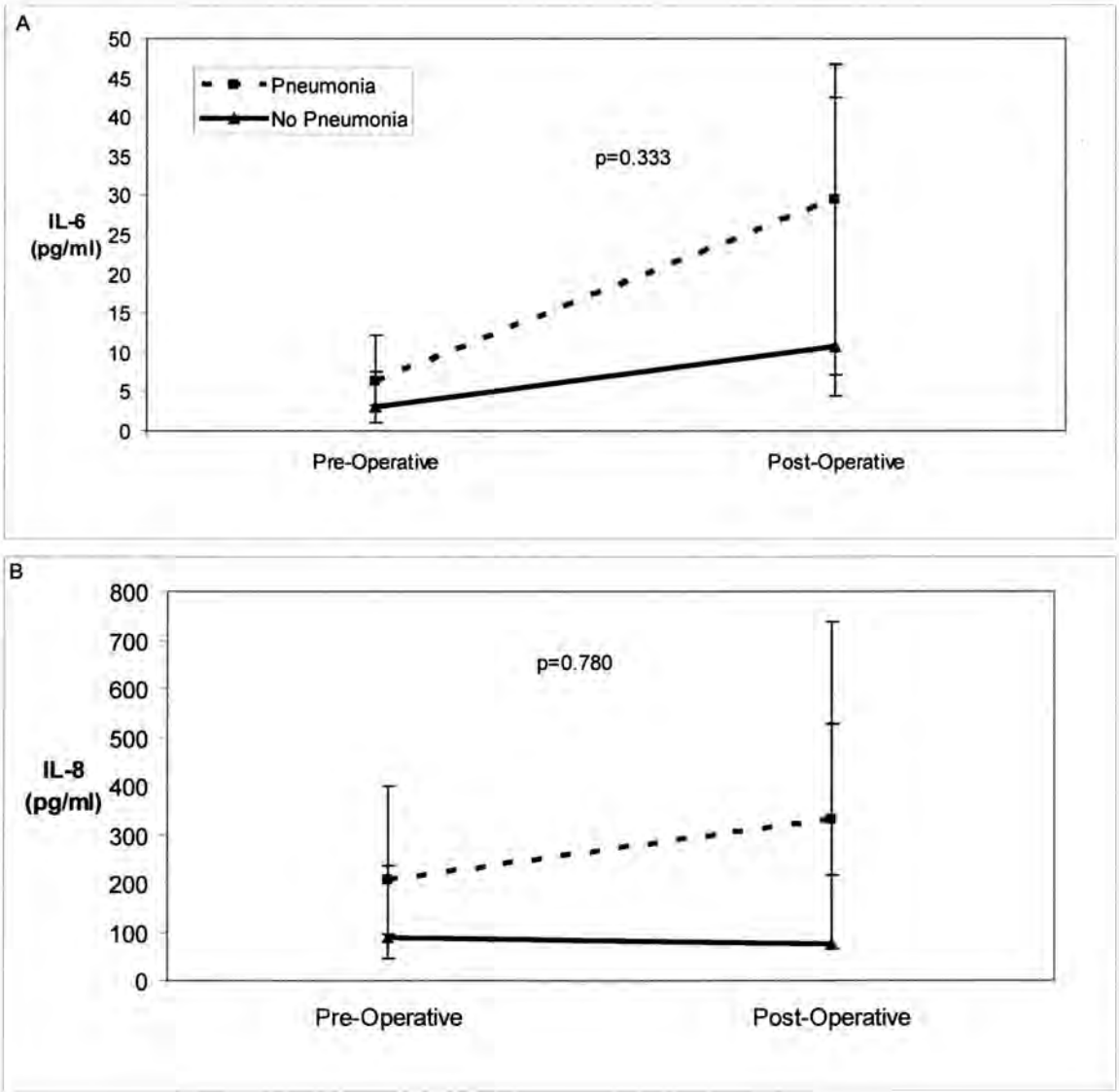


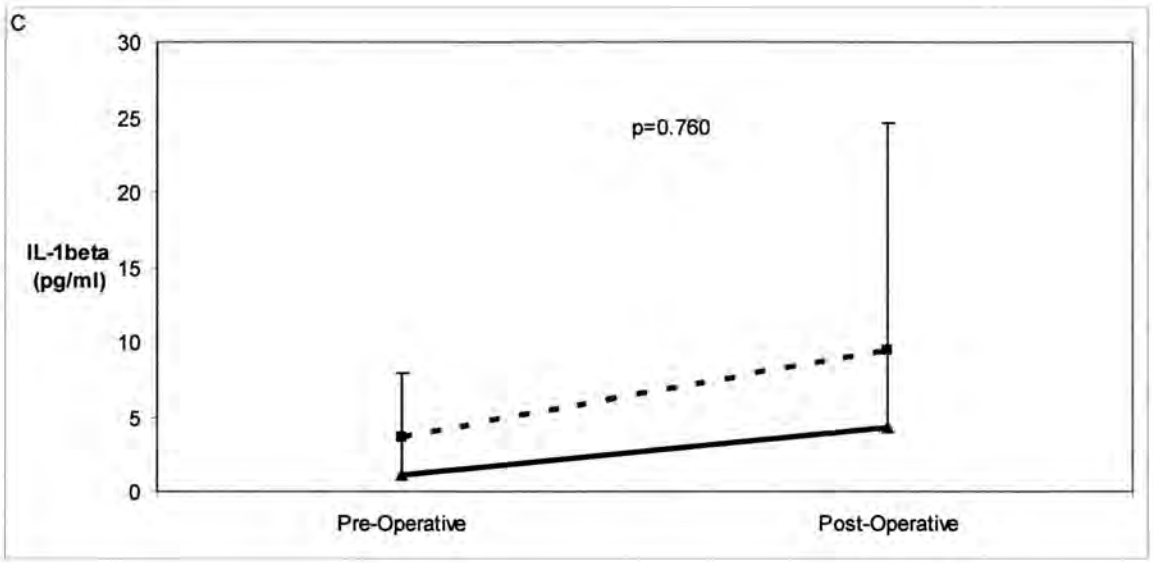
Figure 4.9 Pre-Operative BAL Cellular Concentration was Higher in Patients Subsequently Developing Post-Operative Pneumonia. A different lobe of the contra-lateral lung to the culprit lesion was sampled on each occasion. The data was not normally distributed thus the median pre-operative BAL cellular concentration is shown together with interquartile ranges. The data was $\log_{10}$ transformed and the two groups compared using a two sample t-test. Post-operative pneumonia patients had a higher pre-operative BAL cellular concentration (geometric mean ratio 2.07 p=0.012). n=39.

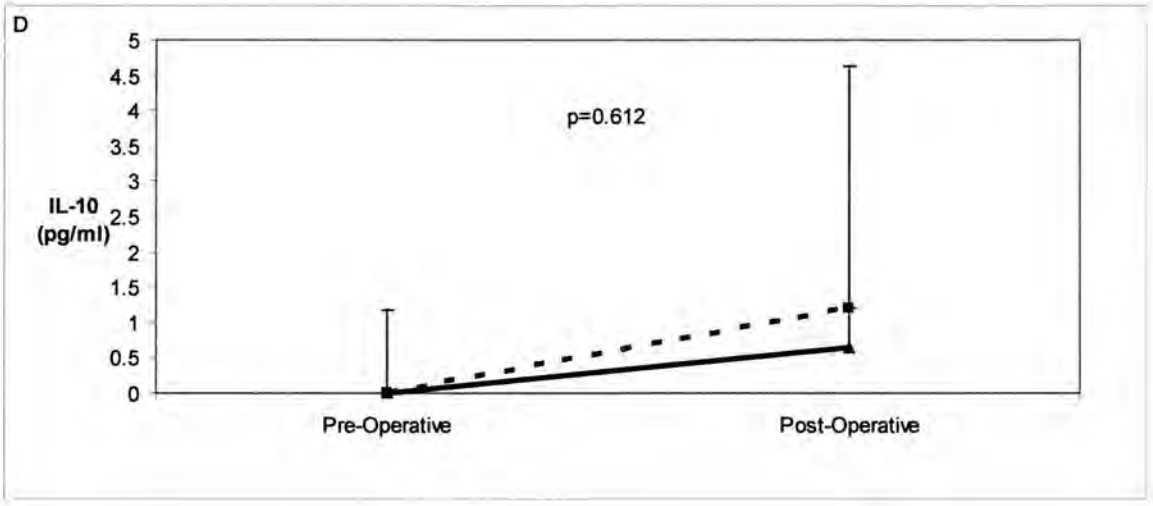
Cell Type	No Pneumonia Pre-Op. Median % (IQR)	No Pneumonia Post-Op. Median % (IQR)	Pneumonia Pre-Op. Median % (IQR)	Pneumonia Post-Op. Median % (IQR)	p-value for Comparison of Change
Macrophages/Monocytes	91 (76-96)	88 (81-92)	94 (53-97)	81 (37-87)	0.771
Neutrophils	4 (2-21)	9 (3-17)	5 (1-46)	15 (11-60)	0.988
Lymphocytes	2 (1-5)	3 (1-5)	1 (1-2)	2 (1-3)	0.146
Other	2 (0)	0 (0)	0 (0)	2 (0)	1.000

Table 4.6 A Comparison of BAL Differential Cell Count for Post-Operative Pneumonia and No Pneumonia Patients. A different lobe of the contra-lateral lung to the culprit lesion was sampled on each occasion. A cytospin was performed of the pellet of cells obtained after centrifugation. Differential cell count was undertaken after Giemsa staining. The data was not normally distributed thus the median values are shown above together with the interquartile range (IQR) in brackets. The data was $\log_{10}$ transformed prior to undertaking a two sample t-test comparing pneumonia and no pneumonia groups. No statistically significant differences were found. n=33.

Figure 4.10 Post-Operative Change in BAL Cytokines for Post-Operative Pneumonia and No Pneumonia Patients. BAL was undertaken prior to surgical staging from the contralateral lung to the culprit lesion and from another lobe of the contralateral lung immediately after completion of lung resection. Cytokines in the supernatant were measured. [Panels A-D] The data for IL-6, IL-8, IL-1β and IL-10 were not normally distributed and are thus reported as medians with the error bars representing the interquartile ranges. The data for IL-12 and TNF-α were generally below the limits of detection and are not shown. Post-operative change was calculated and the two groups compared using a two-sample t-test after $\log_{10}$ transformation. No statistically significant differences were found between the groups. ANCOVA modelling also did not reveal a significant difference between pneumonia and no pneumonia groups (p value shown on charts). n=35.







Monocyte Subsets and Ex-vivo Monocyte Cytokine Response to LPS

Chapter III revealed that there was a relative decrease in the $CD14^{-}$ $CD16^{++}$ monocytes due to an expansion in the absolute numbers of $CD14^{++}CD16^{-}$ and $CD14^{++}CD16^{+}$ subsets. This pattern was found in both pneumonia and no pneumonia patients with no statistically significant differences between the groups found (data not shown).

Six cytokines were measured in the supernatant of isolated monocytes stimulated with LPS. IL-12 levels were found to be significantly lower pre-operatively in patients whom

subsequently developed a post-operative pneumonia compared to no pneumonia (geometric mean ratio 1.19 95% CI 1.00-1.27 p=0.008, Figure 4.11). There was no other pre-operative difference between the groups for the other measured cytokines (IL-1β, IL-6, IL-8, IL-10 and TNF-α).

Figure 4.12 details the post-operative changes between the groups. Significant differences were found in IL-10, IL-6 and IL-8 responses for pneumonia compared with no pneumonia patients. Levels of IL-6 and IL-8 were higher at all time points in patients with pneumonia. Median levels of IL-6 increased sharply at 6hrs then continued to increase reaching statistical significance at 24hrs (geometric mean ratio 1.50 95% CI 1.02-2.19 p=0.040) and 48hrs (geometric mean ratio 1.20 95% CI 1.02-2.19 p=0.014). This contrasted with no pneumonia patients whose IL-6 levels peaked at 6hrs post-operatively before falling.

Levels of IL-8 increased sharply at 6hrs in pneumonia patients and then rose only slightly higher at 24hrs and 48hrs. Although levels in the no pneumonia group were lower at all time points, there was a median increase at 48hrs. Despite this, the difference between the groups was significant at 48hrs (geometric mean ratio 1.20 95% CI 1.06-1.34 p=0.004). The profile for IL-10 in pneumonia patients was marginally lower than no pneumonia patients until after 6hrs where a steady increase in pneumonia patients and fall in no pneumonia patients led to a significant difference at 24hrs (geometric mean ratio 1.24 95% CI 1.05-1.47 p=0.014). No significant difference was found between the groups for IL12-, IL-1β or TNF-α response. ANOVA modelling did not identify a significant difference between the groups for any measured cytokine.

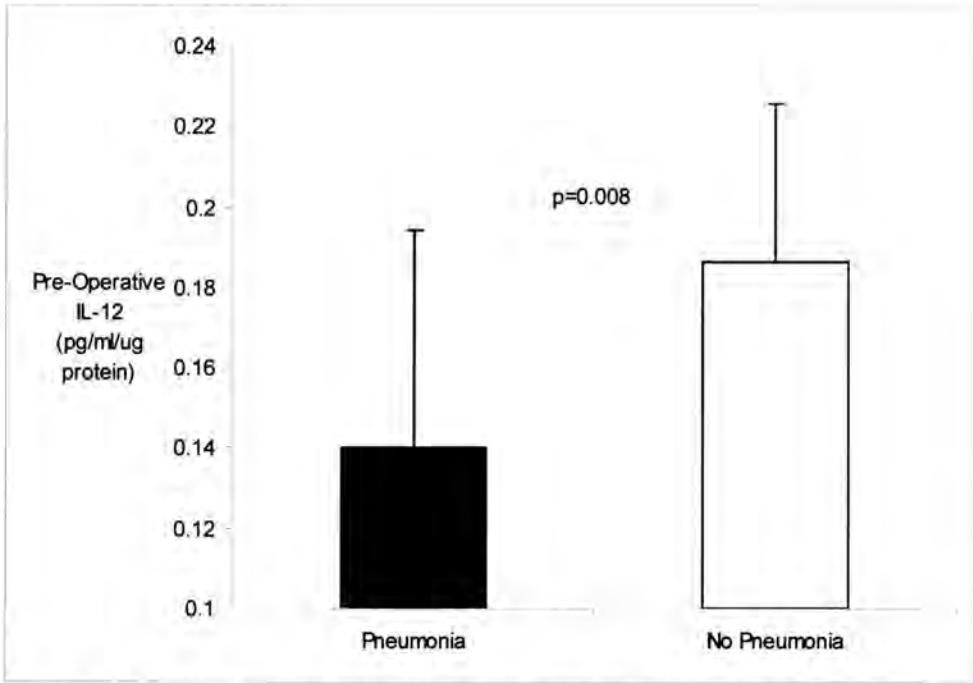
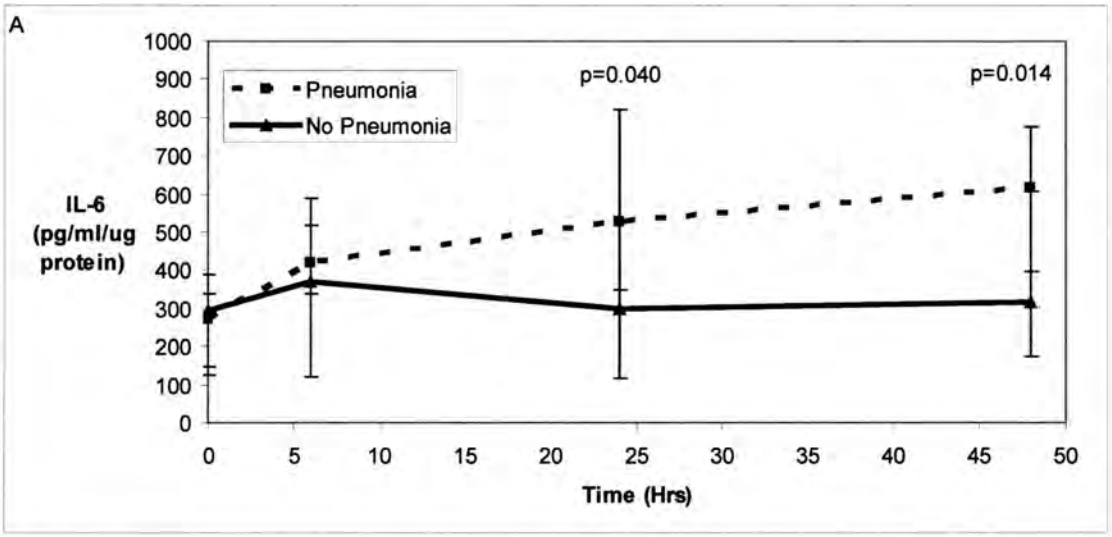
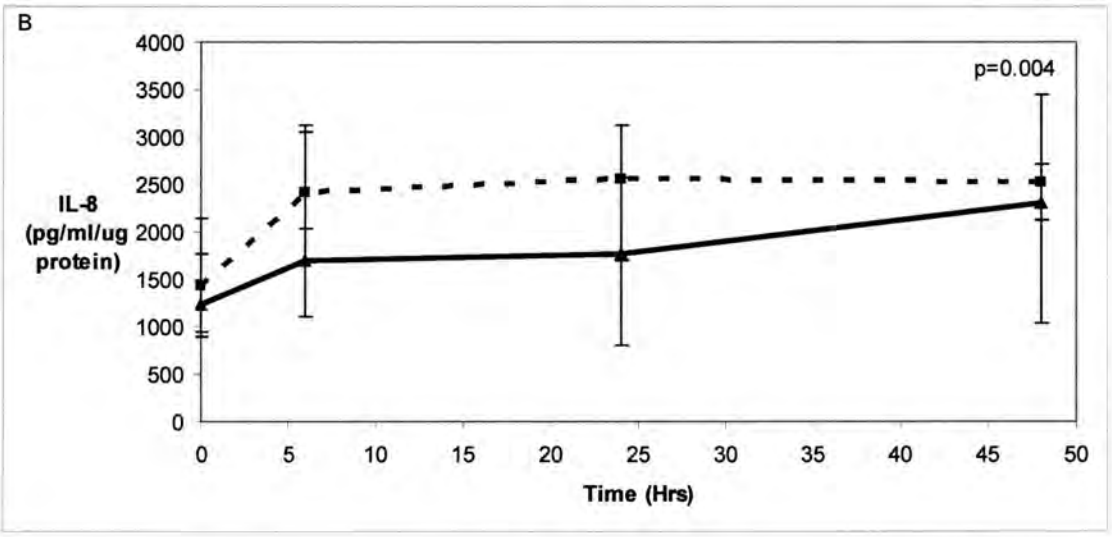
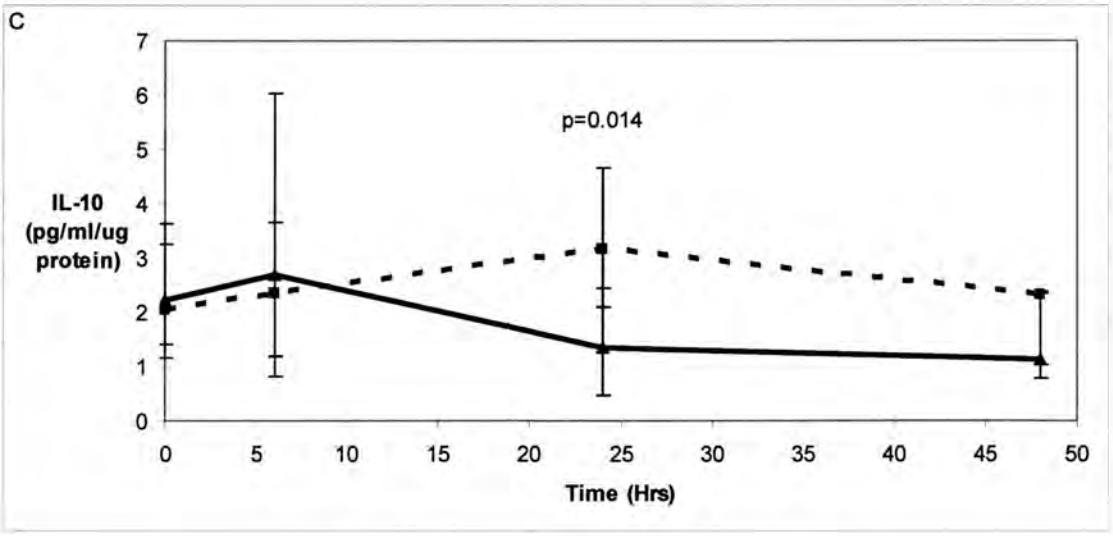


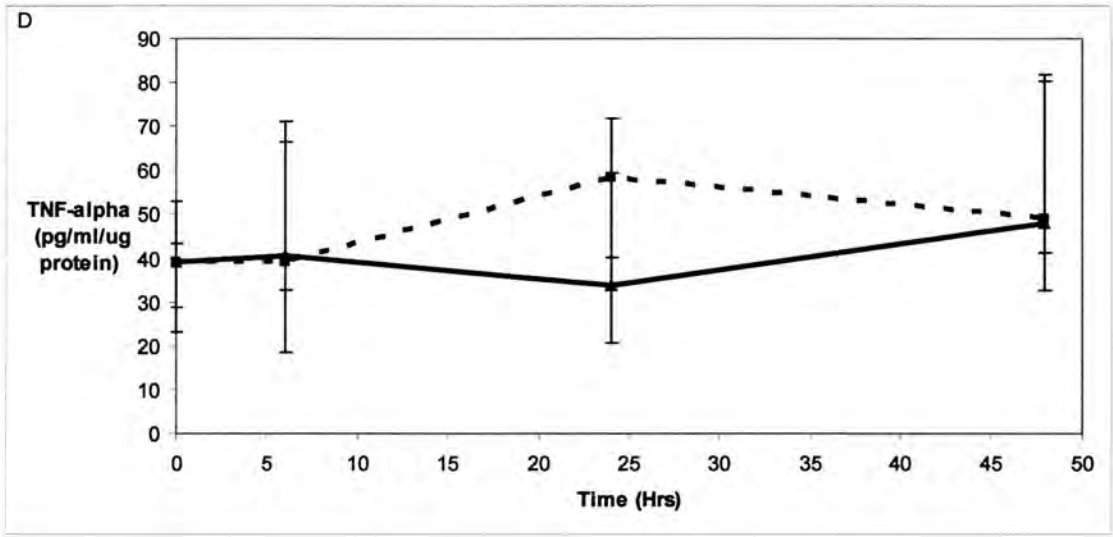
Figure 4.11 Pre-Operative IL-12 was Lower in Patients Subsequently Developing Post-Operative Pneumonia. Monocytes were isolated from whole blood using dextran sedimentation, Percoll extraction and positively selected from the mononuclear layer using $CD14^+$ magnetically labelled beads. Cells were stimulated with 100ng LPS for 18hrs and the supernatant frozen at -80°C until later cytokine analysis. The average of two wells of monocytes stimulated with LPS was calculated and the data adjusted for total protein in each well. IL-12 levels were found to be significantly lower pre-operatively in patients whom developed a post-operative pneumonia compared to no pneumonia (geometric mean ratio 1.19 p=0.008). n=40.

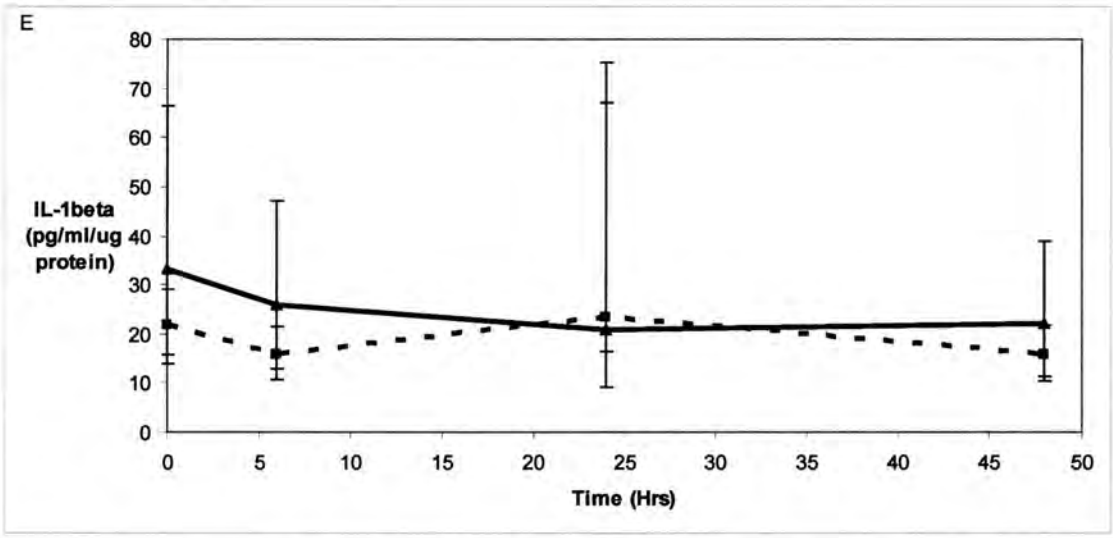
Figure 4.12 Increased Post-Operative IL-6, IL-8 and IL-10 in The Supernatant of Monocytes Stimulated with LPS. Monocytes were isolated from whole blood using dextran sedimentation, Percoll extraction and positively selected from the mononuclear layer using $CD14^+$ magnetically labelled beads. Cells were stimulated with LPS for 18hrs and the supernatant analysed for cytokines. [Panels A-F below] The data was not normally distributed thus the median value is shown. Error bars represent the interquartile ranges. Post-operative change was calculated and the data $\log_{10}$ transformed. There was a general trend for higher cytokine levels in pneumonia patients with statistically significant differences at specific time points shown by the p-value on the charts. There were no statistically significant differences at any time point for IL-12, IL-1β and TNF-α. ANOVA modelling did not reveal any significant difference between pneumonia and no pneumonia groups. n=40 at each time point except t=4 where n=38.

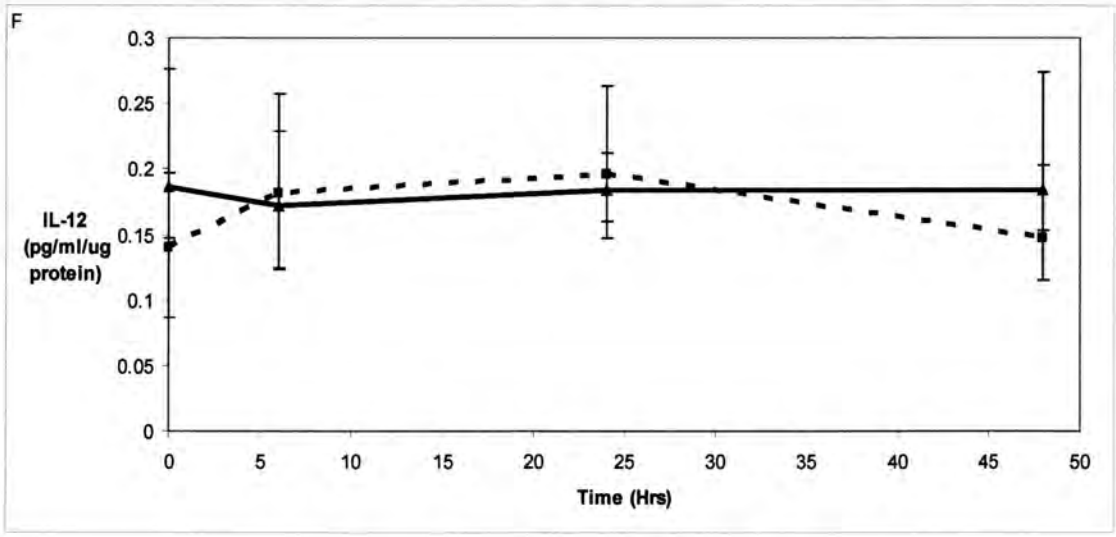

A
Pneumonia
No Pneumonia
p=0.040
p=0.014
IL-6 (pg/ml/ug protein)
Time (Hrs)


B
p=0.004
IL-8 (pg/ml/ug protein)
Time (Hrs)


C
p=0.014
IL-10 (pg/ml/ug protein)
Time (Hrs)


D
TNF-alpha (pg/ml/ug protein)
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
35
40
45
50
Time (Hrs)


E
IL-1beta (pg/ml/ug protein)
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
35
40
45
50
Time (Hrs)


F
IL-12 (pg/ml/ug protein)
0.3
0.25
0.2
0.15
0.1
0.05
0
0
5
10
15
20
25
30
35
40
45
50
Time (Hrs)

Exhaled Nitric Oxide

Exhaled nitric oxide was evaluated as a non-invasive measure of airway inflammation after lung resection which could potentially identify patients at risk of pneumonic lung processes early. The general post-operative trend for initial reduction in exhaled nitric oxide was found for both pneumonia and no pneumonia groups with no statistically significant difference found at any time point (Figure 4.13). Pneumonia patients did however show a sharper initial reduction in exhaled nitric oxide before a median increase in FENO at 48 hours. Using the two-compartment model of airway dynamics and measuring exhaled nitric oxide at three flow rates, alveolar nitric oxide was estimated (Figure 4.14). The trend broadly followed FENO except the no pneumonia patients had a small increase in alveolar NO at 6 hours compared to a decrease for pneumonia patients. No statistically significant differences were found.

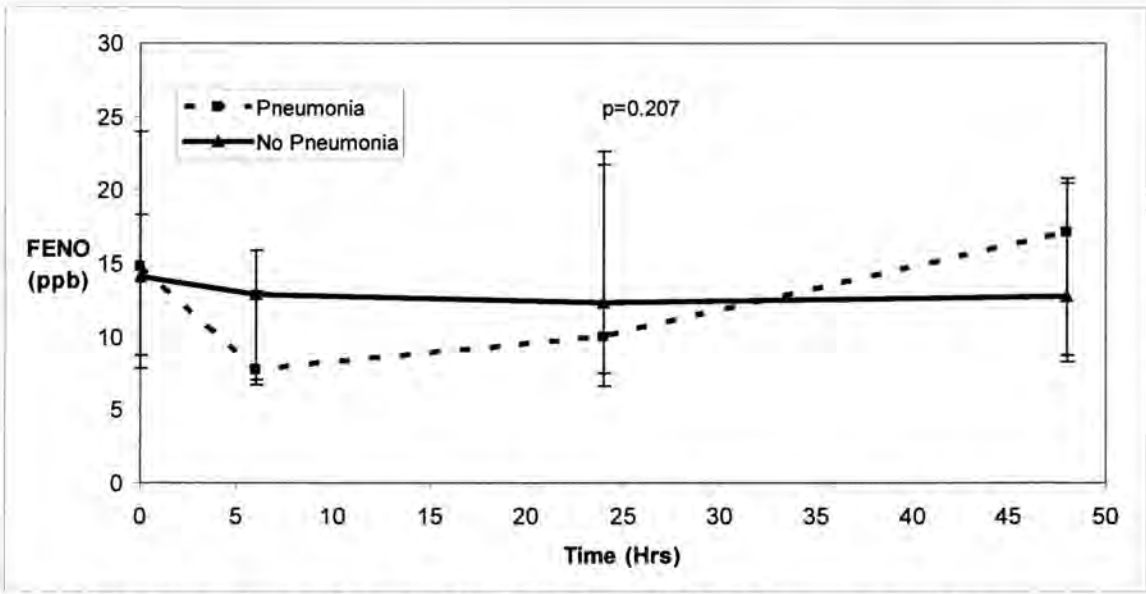


Figure 4.13 Comparison of Exhaled Nitric Oxide (FENO) in Pneumonia and No Pneumonia Patients. The data were not normally distributed thus median values from the Niox Flex are shown. Error bars represent the interquartile ranges. Post-operative change was calculated and the groups compared using the two-sample t-test. No statistically significant differences were found. ANOVA modelling also found not difference between the groups (p value on chart). n=40 pre-operatively, n=31 6hrs post-operatively, n=32 24 and 48 hrs post-operatively.

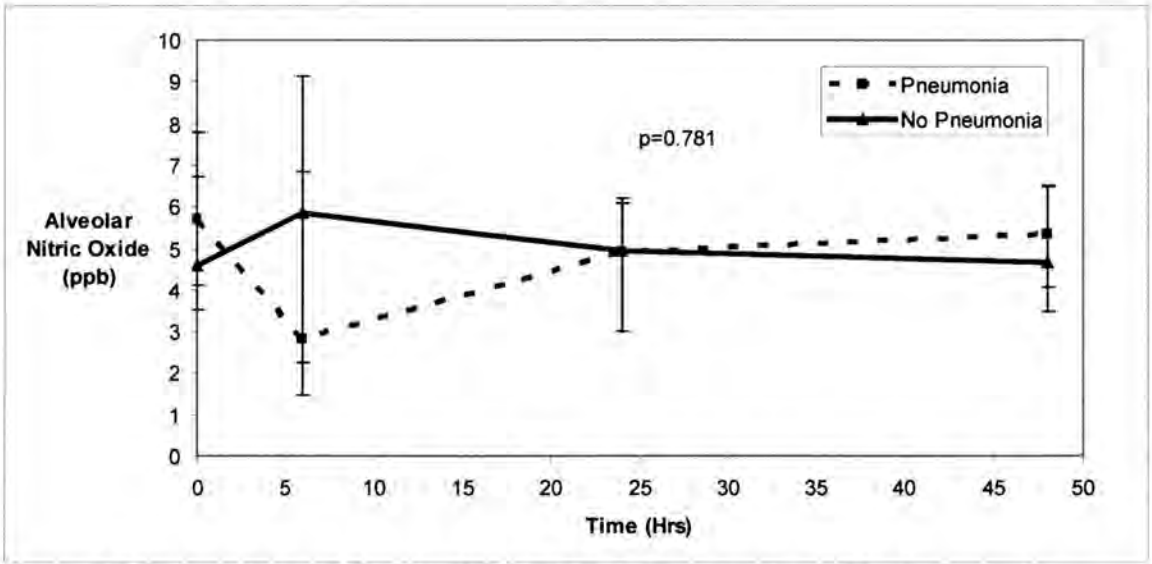


Figure 4.14 Comparison of Alveolar Nitric Oxide in Pneumonia and No Pneumonia Patients. The data were not normally distributed thus median derived values are shown with error bars representing the interquartile range. They were calculated using the two-compartment model of airway dynamics and variable flow rates of 30ml/second, 50mls/second and 100mls/second. Post-operative change was calculated and the two groups compared after $\log_{10}$ transformation using the two-sample t-test and ANOVA modelling. No statistically significant differences were found. ANOVA p value is shown on the chart. N=39 pre-operatively, n=29 6hrs post-operatively, n=27 24hrs post-operatively and n=30 48 hrs post-operatively.

Biomarkers Associated with Post-Operative Pneumonia

The above analysis identified a number of innate immune markers that were statistically significant between patients developing post-operative pneumonia and those not. These are summarised in Table 4.7. The capacity of these individual biomarkers to distinguish pneumonia patients from no pneumonia patients was tested. Three pre-operative and two post-operative parameters appeared to retain significance in predicting pneumonia. Pre-operatively, a blood neutrophil count above 5.04×10^9/L had a relative risk (RR) for pneumonia of 3.3 (95% confidence interval (CI_{95}) 1.1-10.1), and a BAL cell count of greater than 1.04×10^5/ml had a RR of 3.4 (CI_{95} 1.3-9.0), whilst LPS-stimulated monocyte secretion of IL-12 of less than 0.15 pg/ml/μg protein had a RR of 3.0 (CI_{95} 1.2-7.3). At 24 hours post-operatively, LPS-stimulated release from monocytes of IL-10

greater than 1.99 pg/ml/μg protein (RR 4.1, CI_{95} 1.3-12.3) and IL-6 greater than 414 pg/ml/μg protein (RR 3.1, CI_{95} 1.2-8.1) were predictive of pneumonia.

Marker	Pneumonia	No Pneumonia	p-value
Pre-Operative white cell count in whole blood ($x10^9$/l)	9.1 (8.4-11.2)	7.9 (6.7-9.5)	0.007
Pre-Operative blood neutrophils ($x10^9$/l)	5.8 (5.1-6.9)	4.7 (4.1-5.7)	0.015
Pre-Operative BAL white cell count (millions/ml)	0.14 (0.1-0.2)	0.08 (0.0-0.1)	0.012
Serum IL-8 (48Hrs) (pg/ml)	17.4 (13.2-21.1)	14..5 (10.5-16.8)	0.031
Serum TNF-α (48Hrs) (pg/ml)	6.9 (4.0-10.3)	7.5 (5.3-9.2)	0.023
Monocyte supernatant IL-12 (pre-operative) (pg/ml/μg)	0.1 (0.1-0.2)	0.2 (0.1-0.3)	0.008
Monocyte supernatant IL-10 (24Hrs) (pg/ml/μg)	3.2 (2.1-4.7)	1.4 (0.5-2.4)	0.014
Monocyte supernatant IL-6 (24Hrs) (pg/ml/μg)	525.4 (347.3-822.6)	297.6 (114.6-531.6)	0.040
Monocyte supernatant IL-6 (48Hrs) (pg/ml/μg)	616.0 (396.5-778.0)	315.3 (175.5-606.7)	0.014
Monocyte supernatant IL-8 (48Hrs) (pg/ml/μg)	2525.2 (2127.2-3447.6)	2297.9 (1043.0-2719.3)	0.040

Table 4.7 Biomarkers associated with the development of post-operative pneumonia. Data are presented as medians with IQRs in brackets. Statistical analysis was by two sample t-test. N=40 for all comparisons except pre-operative BAL, serum and monocyte supernatant experiments at 48hrs, where n=38. All monocyte supernatant values refer to monocytes stimulated *ex vivo* with lipopolysaccharide.

V - DISCUSSION

This study has demonstrated that there are early and profound changes in innate immune function following major lung resection, many of which are reduced following VATS lobectomy compared with lung resection undertaken via conventional thoracotomy. Specifically, VATS surgery induced a smaller increase in serum cytokines, and was associated with higher serum albumin counts together with reduced IL-10 release from LPS-stimulated monocytes at 6hrs post-operatively compared with thoracotomy. Further, the magnitude of the innate immune response appears associated with the risk of post-operative pneumonia. A relative leucocytosis in blood or BALF pre-operatively and heightened responsiveness of blood monocytes to LPS early in the course of surgery, seemed particularly associated with later development of pneumonia. Together, these findings suggest that innate immune function may influence the pathogenesis of post-operative pulmonary inflammation and could potentially aid stratification of post-operative risk in the future enabling targeted prophylactic or therapeutic strategies.

Post-Operative Systemic Inflammatory Reponses - VATS versus Thoracotomy

VATS lobectomy was associated with lower release of post-operative serum cytokines; pro-inflammatory IL-6 and IL-8 together with anti-inflammatory IL-10 levels were all significantly lower (Figures 3.1-3.3). Although there was no significant post-operative change in TNF-α, higher levels were associated with thoracotomy patients (Figure 3.4). There was no post-operative change or difference found between the groups for IL-1β or IL-12 (Figure 3.4). These findings are consistent with earlier published studies. Yim et al. identified reduced secretion of IL-6, IL-8 and IL-10 following VATS lobectomy in patients with stage I NSCLC compared with thoracotomy (Yim et al., 2000). No post-operative change or difference was found for IL-1β and TNF-α. Craig et al. also identified lower IL-6 and CRP release following VATS lobectomy compared with thoracotomy (Craig et al., 2001). Nagahiro et al. found reduced IL-6 secretion after

VATS lobectomy for stage I NSCLC but interestingly no difference for IL-8 and IL-10 (Nagahiro et al., 2001). The reason for this discrepancy is unknown. Interestingly, the reported IL-6 levels in Nagahiro's study were much lower than those described here or in other studies. Since IL-6 release is proportionately higher than IL-8 and IL-10 release, this may explain why they found no significant difference between surgery types for these measured cytokines.

The body's response to surgical trauma is complex and involves the interaction of various immunological and physiological systems including humoral and cellular immunity, endocrine processes and neural stimulation (Walker et al., 2007). Immunomodulating agents such as cytokines play an important role in cellular messaging and the inflammatory cascade in addition to chemokines, growth factors, lipid and complement. Previous studies of thoracic surgical patients have described an association between elevated post-operative IL-6 and the systemic inflammatory response syndrome (SIRS) (Takenaka et al., 2006) and between post-operative serum IL-6/IL-8 and post-operative sepsis (Yamada et al., 1998). IL-6 is indeed known to generate pyrexia, activate the hypothalamic pituitary axis and influence the hepatic acute-phase response (Raeburn et al., 2002). However, there are many such proteins released following surgical trauma with immunoregulatory roles. The requirement for a delicately regulated balance between pro- and anti-inflammatory responses to pathological insults is well described (Hotchkiss et al., 2003), and the implication is that dysregulation of this balance may result in adverse outcomes.

It is important to appreciate that the presence of malignancy per se may also influence cytokine release. Thus, baseline measurements of IL-12, TNF-α and IL-6 were marginally higher in pre-operative lung cancer patients compared to an age-matched, mixed gender control group (Table 3.7), suggesting an underlying pro-inflammatory state in cancer patients. Higher serum cytokine levels in lung cancer patients have previously been reported for example, TNF-α (Atwell et al., 1998) and IL-6 (Koh et al., 2012).

One important limitation of the comparison between lung resection performed via VATS and thoracotomy in this study is that patients were not randomised into either group. Surgery was performed whenever possible utilising the VATS approach. VATS targeted early stage peripheral lung tumours. Thus, there were significantly more adenocarinomas in the VATS groups compared to more squamous cell carcinomas in the thoracotomy group (Table 3.2). Further, there were more stage IA tumours in the VATS group (Table 3.3). Moreover, the actual operation performed differed between the groups: the VATS group included lobectomy and segmentectomy whereas sleeve resections, a chest wall resection and two pneumonectomies were performed in addition to lobectomy in the thoracotomy group (Table 3.4). The impact of these details on the inflammatory response is uncertain. Although the numbers were small, no significant differences in serum cytokines were found when comparing lobectomy via thoracotomy and other lung resections via thoracotomy. Despite the lack of randomisation, the groups were well matched in terms of age and BMI (Table 3.1). More males however were in the thoracotomy group and there was a non-significant trend for higher FEV_1 in the VATS group together with a non-significant higher Thoracoscore.

It may also be a criticism that three patients in the thoracotomy group underwent initial video-thoracoscopic inspection whereupon VATS resection was deemed technically inappropriate leading to conversion to conventional thoracotomy. These operations would have been slightly longer than if thoracotomy was performed from the outset, with a potential, albeit small, impact on the inflammatory response. The conversions however took place relatively soon after surgery commenced and the study was designed to compare the VATS approach with thoracotomy.

VATS resection was associated with higher levels of serum albumin (Figure 3.7). Serum albumin, produced by the liver, is reduced as part of the acute phase response and is thus a 'negative' acute phase protein (Ritchie et al., 1999). This is thought to allow amino acids to be used for producing positive acute phase proteins more

efficiently. The lower levels are consistent with a reduced systemic inflammatory response after VATS resection although were higher pre-operatively as well in the VATS group. Low pre-operative serum albumin has been previously associated with post-operative complications after lung resection (Amar et al., 2007 and Ferguson et al., 2008). Post-operative hypoalbuminaemia has also been associated with poorer surgical outcomes in off-pump coronary artery bypass surgery (Lee at al., 2008).

The clinical relevance of the reduced serum inflammatory response with VATS lung resection is currently unclear. There is growing evidence that reduced post-operative inflammation may lead to less peri-operative morbidity (Amar et al., 2007 and Shaw et al. 2005) and certainly I have demonstrated heightened immune responses are associated with post-operative pneumonia. However, no significant difference was found in post-operative complications between the two surgical groups in this relatively small study. Theoretically, reduced peri-operative immunosuppression with VATS should lead to reduced risk of metastasis and recurrence. A meta-analysis found a reduced systemic recurrence rate and improved 5-year mortality rate with VATS (Yan et al., 2009). A more recent meta-analysis confirmed these findings in stage I lung cancer (Li et al., 2012). The only other meta-analysis published on the subject also found reduced mortality when non-randomised studies were included (Cheng et al., 2007). A recent study linked higher IL-6 levels on day 1 post-operatively with a higher risk of early post-operative recurrence, even if the resection was considered complete (Kita et al., 2011).

Ideally, a large multi-centre randomised controlled trial would be conducted measuring immune function, short- and long-term morbidity and mortality after lobectomy via VATS and thoracotomy. Such a study is unlikely to happen however due to the expense and logistics of measuring immune function as well as the difficulty of established VATS centres randomising patients into a thoracotomy group, with patient preference highly likely to be in favour of the minimally invasive approach.

BALF

BAL was used in this study to determine acute inflammatory responses to surgery at a local, pulmonary level. Previous studies have described an increase in pulmonary inflammation early in the course of thoracic surgery using serial BAL (Schilling et al., 2005, Zingg et al., 2010 and Breunig et al., 2011). While informative, these studies are confounded by the low volumes of saline instilled, reducing sample quality (Haslam et al., 1999) and the close proximity of the pre- and post-operative BAL sampling protocols. BAL itself is known to induce a brisk inflammatory response, recovering by approximately 72 hours, which may impact on specimens obtained by repeated local sampling (Von Essen et al., 1991, Huang Y-C et al., 2006 and Terashima et al., 2001). Nevertheless, a separate study conducted by myself with seventeen patients, confirmed that in the context of surgery, BAL does not induce a significant additional systemic inflammatory response (appendix).

The pre-operative sample in this study was taken before mediastinoscopy (after induction of anaesthesia) from the contra-lateral lung to the tumour, while the post-operative sample was taken at least one week later from another lobe of the contra-lateral lung, immediately after lung resection whilst the patient was still anaesthetised in theatre (Table 3.11). The higher protein content (Figure 3.16), increased cellular content (Figure 3.15 and Table 3.12) and cytokine composition (Figure 3.17) confirm that the lung contralateral to the operated tumour is inflamed immediately after thoracic surgery.

This early pro-inflammatory effect is most likely attributable to one lung ventilation, used in all patients in this study, and previously shown to induce inflammatory effects in the lung (Moloney et al., 2004). OLV may result in inflammation due to high FiO_2, leading to the generation of ROS, high tidal volumes and pulmonary capillary stress failure due to more pulmonary artery blood entering the non-collapsed lung (Baudouin, 2003). One study demonstrated reduced levels of TNF-α and sICAM-1 in the ventilated lung after thoracic surgery in patients ventilated with a tidal volume of 5mls/kg

compared with 10mls/kg (Schilling et al., 2005). Others have found no difference in pulmonary cytokines when using lower tidal volumes (Wrigge et al., 2004) although this study was based on tracheal aspirates rather than BAL which are less reflective of alveolar inflammation. Certainly, numerous animal studies and clinical trials have found ventilation with tidal volumes slightly above physiological levels can rapidly cause acute lung injury (Dos Santos et al., 2000).

The higher protein and cellular content observed post-operatively are consistent with earlier published studies in patients undergoing thoracic surgery. Schilling et al. performed BAL immediately before major thoracic surgery, including lung resection, and at two time points after surgery from the dependent, ventilated lung (Schilling et al., 2005). The total number of cells, protein and albumin counts all increased after surgery. Schilling et al. also found higher levels of IL-8 post-operatively which has been confirmed in the present study together with the work of Cree et al. and Zingg et al. in oesophagectomy patients requiring one-lung ventilation (Cree et al., 2004 and Zingg et al., 2010). IL-8 is an important chemotactic peptide primarily for neutrophils as well as other granulocytes, inducing physiological responses such as phagocytosis. Levels in BAL have been shown to be increased in a range of pathological processes including ARDS, sepsis and multi-organ failure (Schuttte et al., 1996). Indeed, although neutrophils have beneficial anti-microbial effects they may prove detrimental to the host with release of toxic products including elastase, leukotrienes and free radicals into the alveolar space (Boutten et al., 1996).

No patient developed clinical ALI/ARDS in this study. The only pulmonary parameter associated with subsequent development of pneumonia was a relatively high pre-operative leucocyte count in BALF, indicating perhaps a pro-inflammatory tendency or underlying subclincial infection/colonisation. Importantly, no relationship was demonstrated between BALF IL-8 levels and post-operative pneumonia in the present study, with BAL sampled immediately post-operatively. Whether differences emerge at 24hrs or 48hrs post-operatively would be an interesting enquiry. IL-8 and IL-1β have

previously been shown to be important in diagnosing ventilator-associated pneumonia in non-surgical, critically ill patients (Conway Morris et al., 2010).

Higher levels of pro-inflammatory IL-6, IL-1β and a small increase in anti-inflammatory IL-10 was also found in this study. IL-6 was found to be increased in the non-operated lung of lung cancer patients by Breunig et al. together with anti-inflammatory IL-1RA (Breunig et al., 2011). This group also analysed BAL from the operated lung in the same patient and found, perhaps unsurprisingly, higher IL-6 levels in the operated lung. Indeed, handling and dissection of lung tissue together with stapling devices across lung, bronchus and vascular tissue is likely to induce an acute inflammatory response. Interestingly, in oesophagectomy patients, where direct lung trauma to the non-ventilated lung is avoided, BAL cytokine inflammatory responses are greater in the dependent, ventilated lung (Zingg et al., 2011).

No change in TNF-α was found in this study which contrasts with the findings of Schilling et al. who found an increase in TNF-α together with a reduction in IL-10 post-operatively. The reason is unknown although may be related to differences in BAL sampling methodology. This study also found no significant differences in any BALF analysis between VATS and thoracotomy patients. This may be explained by the fact that samples were taken from the non-operated lung and both groups of surgical patients underwent standardised OLV.

Cellular Immune Responses

Lung resection was associated with a monocytosis (Figure 3.5B) due to an increase in classical and intermediate monocytes in peripheral blood (Table 3.10), with no significant difference identified between VATS and thoracotomy. Similar numerical changes have been described after stroke (Urra et al., 2009) with a relative expansion in intermediate monocytes and relative reduction in non-classical monocytes. A relative reduction in non-classical monocytes has also been described following coronary artery

bypass surgery in on- and off-pump surgery suggesting the effect was due to surgery and not the cardiopulmonary bypass machine (Wehlin et al., 2005).

Conversely, septic patients have been shown to expand the non-classical pool of monocytes (Fingerle et al., 1993) which are characterised by higher MHC II expression and higher production of TNF-α after stimulation with Toll-like receptor ligands (Ziegler-Heitbrock et al., 2010). A host of other inflammatory and infectious diseases are also associated with an expansion in the non-classical pool of monocytes including atherosclerosis, Kawasaki disease and bacterial infections (Ziegler-Heitbrock, 2007). The last blood sample in this study was taken at 48hrs and it is tempting to speculate that the expansion in the intermediate pool could eventually expand the non-classical pool, as seen in septic patients (Fingerle et al., 1993). There was however no association between post-operative pneumonia and changes in monocyte subset populations.

The pattern of increased LPS-induced cytokine secretion from monocytes post-operatively (Figure 3.12) is broadly similar to that described in a smaller study of mononuclear cells from (non-thoracic) surgical patients (Cabié et al., 1992), though we did not observe the increase in IL-1β described in that study. Release of the anti-inflammatory cytokine IL-10 from monocytes was initially increased, but was blunted by 48 hours, implying a net pro-inflammatory response of monocytes by that time. The novel finding that enhanced early post-operative monocyte responsiveness maps to increased risk of post-operative pneumonia (Table 4.7) suggests a potentially important place for the monocyte in pneumonia pathogenesis. Certainly, it has been shown that in established sepsis, there is down-regulation of monocyte HLA-DR expression and reduced ability to produce TNF-α in response to LPS (Döcke et al., 1997).

Interestingly, reduced IL-12 release from LPS-stimulated monocytes retrieved pre-operatively provided another strong association with later pneumonia. In mice, IL-12 has been shown to be protective against pneumonia induced by *Klebsiella pneumoniae* (Greenberger et al., 1996) and *Pneumocystis carinii* (Ruan et al., 2008). Conversely, it

appears to impair host defence against *Mycoplasma pneumoniae* (Salvatore et al., 2008). These findings compare with those of Hensler et al. who noted reduced IL-12 release from LPS-stimulated monocytes in patients progressing to post-operative sepsis after major elective gastrointestinal surgery (Hensler et al., 1998). It is important to note that I did not find surgery to significantly impact on IL-12 levels and that the association with pneumonia was only apparent with pre-operative levels of the cytokine.

Lower levels of IL-10 production from monocytes stimulated with LPS were found with VATS surgery at 6 hours post-operatively indicating less disturbance of peri-operative immunity (Figure 3.12G). Nevertheless, no other differences were found with the other measured cytokines produced ex-vivo from monocytes.

It is noteworthy that there were relatively high levels of IL-8 in normal monocyte cultures (non-LPS) from surgical patients and healthy controls (Table 3.8). Positive selection with anti-CD14-labelled microbeads should not lead to signal transduction since the essential co-receptors, such as TLR-4 and MD-2 are absent (Kitchens, 2000). The cells were however cultured with patient serum from the relevant time point in order to simulate the in vivo cytokine environment in which the cells would normally function. This would have influenced cytokine production although doubtful to any significant extent in comparison to LPS, particularly in the control group.

While monocytes generally displayed a pro-inflammatory phenotype after lung resection, blood neutrophils, although increased in number (Figure 3.5A), were associated with a minor reduction in phagocytic capacity for zymosan (Figure 3.11). There was a small increase in *E. Coli* phagocytosis at 48 hrs post-operatively although no significant overall change post-operatively on ANOVA modelling. A small, but statistically significant, reduction in phagocytic capacity has also been described after abdominal surgery (Kawasaki et al., 2007). It seems unlikely that the small reduction in neutrophil phagocytosis was clinically important, as there was no association with post-operative pneumonia (Figure 4.8). In previous studies in patients admitted to the ICU it

was shown that impaired neutrophil phagocytosis was associated with increased risk of subsequent ICU-acquired infection, but the level of phagocytic impairment was far greater in that cohort (Conway Morris et al., 2011).

No difference in post-operative neutrophil phagocytic capacity was found between VATS and thoracotomy patients. Previous studies have shown that minimally invasive surgery may affect cellular immunity less than conventional open surgery. Craig et al. showed ROS in neutrophils increased post-operatively in thoracotomy but not VATS patients although actual phagocytosis was not assessed (Craig et al., 2001). All patients developed a relative blood lymphopenia post-operatively (Figure 3.5B) but we did not detect any significant difference between the surgical groups consistent with previous research (Leaver et al., 2000). Previous studies have shown reduced suppression of CD4 T cells at two days after VATS resection compared with thoracotomy and natural killer lymphocytes at seven-days post-operatively (Craig et al. 2001).

Exhaled Nitric Oxide

This study found a trend for reduced FENO levels after lung resection which reached significance at 48hrs post-operatively (Figure 3.20). The overall median reduction was small in the order of a couple of parts per billion. The accuracy of measurements was confirmed using two devices, a chemiluminescence analyser and a smaller, portable and cheaper electrochemical sensor. There was excellent concordance of measurements between the devices at the standardised flow rate of 50mls/second (Figures 3.18 and 3.19), which is consistent with a previous published report in asthmatic patients (Menzies et al., 2007).

Clearly a proportion of lung tissue has been removed which would contribute to this reduction, assuming there was no complete bronchial obstruction by tumour of the affected lung/ lobe. However, the contribution of alveolar nitric oxide to total exhaled nitric oxide is usually small. Indeed, alveolar nitric oxide was calculated using variable

exhaled breath flow rates and the principles of the two-compartment model of airway dynamics (Pietropaoli et al., 1999). Essentially, the model divides the airways into two; the lower, alveolar airways and upper, conducting airways. It is known that exhaled NO increases as expiratory flow rates fall. This phenomenon is due to mixing between the lower and upper airways; at fast exhalations alveolar nitric oxide makes a large contribution to total nitric oxide whereas at slow exhalations NO from the conducting airways predominates. Equations describing this mixing, together with the measurement of total exhaled nitric oxide at different expiratory flow rates, allowed the contribution of the lower and upper airways to be calculated. There was no significant overall change in alveolar nitric oxide (Figure 3.21) implying the small overall reduction observed was due to changes elsewhere. The other confounding variable however was air leakage post-operatively which would have impacted on readings obtained by the chemiluminescent analyser. Air leak was present, certainly early post-operatively, in the majority of patients.

It may also be a criticism that the maximal exhaled flow rate was 150mls/sec in this study which is lower than that used in the Pietropaoli paper. However, this was the highest practical flow rate achievable in the majority of patients presenting for lung cancer surgery; patients generally had impaired lung function pre-operatively together with discomfort and air leakage from the lung post-operatively. The flow rates adopted however were in line with the guideline of >100mls/sec for high flow and <50mls/sec for low flow (ATS, 2005).

If it is accepted that exhaled nitric oxide is reduced, potential mechanisms, in addition to air leakage and removal of lung tissue, include down-regulation of constitutive NOS expression, reduced ability to induce NOS2 by inflammatory cytokines and consumption reactions, for example, NO and superoxide forming the potent peroxynitrite (Kobzik L, 2009). Indeed, activated neutrophils, sequestered to the lung in acute inflammation, release superoxide (Baldwin et al., 1986). Further, post-operative

perialveolar haemorrhage would increase NO-haemoglobin binding, reducing exhaled nitric oxide (Brett et al., 1998).

No patients in this study developed clinical ALI/ARDS. Brett and Evans showed significantly lower levels of exhaled nitric oxide in intubated patients with established ARDS compared with a control group of intubated patients prior to cardiac surgery (Brett et al., 1998). Other groups have shown exhaled NO to be reduced after cardiopulmonary bypass surgery (Ishibe et al., 2000 and Cuthbertson et al., 2002) as well as after lung transplantation (Marczin et al., 1997). The reduction in NO found in this study after lung resection is therefore consistent with the findings of these other forms of ischaemia-reperfusion injury and lung injury. Studies evaluating exhaled nitric oxide post-operatively following lung resection are sparse. One paper examined exhaled nitric oxide levels in patients undergoing thoracic surgery with one-lung ventilation (Minami Y et al., 2003). Using an online system in intubated patients, they found that exhaled nitric oxide levels returned to baseline after releasing one-lung ventilation (which had been in place for approximately 3hours). In contrast, the measurements used in this study were from spontaneously breathing, non-intubated patients without the upper conducting airways excluded by an endotracheal tube. Further, the first post-operative measurement of exhaled nitric oxide in this study was 6hrs post-operatively, when inflammatory changes may have had more time to establish.

The clinical implications of the apparent trend for reduced exhaled nitric oxide post-lung resection are unclear. There was no significant difference in exhaled nitric oxide levels in the early post-operative period between patients developing pneumonia or not (Figure 4.13). This is consistent with a study in medical critical care patients which found that early exhaled nitric oxide was not a useful biomarker for later pneumonia (Tadie et al, 2010). Interestingly however nasal NO was found be moderately predictive for ventilator associated pneumonia. A preliminary study suggested an association between different NO patterns and clinical outcome after lung transplantation (Marczin et al., 1997). Others have found elevated levels in patients with acute rejection after

lung transplantation (Silkoff et al., 1998). These findings have not however been confirmed or found real clinical utility.

No statistically significant differences were found at baseline between exhaled NO in patients and an age matched group of healthy controls although there did appear to be a tendency for higher levels in controls (Table 3.13). The reason for the relatively higher levels in controls is unknown although it is tempting to implicate smoking status. Exhaled nitric oxide levels have been shown to be up to 50% lower in smokers compared with non-smokers (Kharitonov et al., 1995). The groups were well matched in terms of current smoking status (Table 3.6) however there were more lifelong non-smokers in the control group compared with ex-smokers in the patient group. It is also known that ex-smokers have lower exhaled NO levels than non-smokers (Malinovschi et al., 2006). Previous work comparing exhaled nitric oxide of lung cancer patients and healthy controls showed higher levels in lung cancer patients from Taiwan (Liu et al., 1998). However, it is important to note that over 70% of the lung cancer patients were non-smokers (20/28) compared with 32% in this study. Further, the exhaled flow rate used was higher at 200mls/sec than the now standard 50mls/sec adopted for this study.

Novel Biomarkers

Overall, a relatively high baseline number of leucocytes in both the circulation and the alveoli, and a relatively high responsiveness of circulating monocytes, may leave the patient susceptible to pulmonary inflammation post-operatively. It is tempting to speculate that patients with a relatively pro-inflammatory tendency are susceptible to post-operative infection, but this hypothesis requires further investigation. While these findings enhance understanding of the biology and possible clinical implications of the innate immune response to thoracic surgery, interpretation of the data requires some caution. Thus, although the clinical characteristics of our cohort are broadly representative of patients undergoing thoracic surgery, the associations with post-operative pneumonia require confirmation. Further, we cannot be certain of the

aetiology of the pneumonia in this study, as our definition did not require microbiological confirmation, and CXR does not have optimal sensitivity and specificity for the detection of pneumonia (Syrjälä et al., 1998). However, our diagnosis of pneumonia was pre-defined and CXRs were interpreted by a pulmonary radiologist blinded to other study results. Eight of the 14 patients also had positive sputum culture as part of their routine clinical care. We can be relatively confident that our 'pneumonia' patients had alveolar inflammation that would be recognised as such by most clinicians. It is also important to note that most of the patients in this study had lung cancer and a burden of tobacco exposure, both of which can influence innate immunity, together with medications including statins. Finally, as with all functional studies using neutrophils and monocytes, we cannot be certain that the *ex vivo* monocyte responsiveness and neutrophil phagocytosis data are representative of biological activity *in vivo*.

Conclusion

In conclusion, this study provides a unique and comprehensive insight into the early and profound effects that major thoracic surgery has on innate immune function. Surgery induces systemic cytokine release and a blood leucocytosis due to neutrophilia and monocytosis. In addition, a relative blood lymphopenia occurs. There is an expansion in the classical and intermediate monocyte pool with an increase in the responsiveness of blood monocytes to LPS together with minor impairment of the phagocytic capacity of blood neutrophils. BALF from the contralateral lung reveals evidence of early acute inflammation and exhaled nitric oxide levels are generally reduced post-operatively. VATS is associated with reduced increments in pro-inflammatory serum cytokines IL-6 and IL-8 and the anti-inflammatory IL-10. Further, VATS is associated with a significantly lower reduction in serum albumin. Ex-vivo stimulation of isolated monocytes with LPS revealed lower levels of anti-inflammatory IL-10 six hours post-operatively in VATS patients.

Post-operative pneumonia occurred in 35% of patients. Pre-operatively, relatively high numbers of leucocytes in blood and BALF are associated with post-operative pneumonia, as is relatively low secretion of IL-12 from LPS-stimulated monocytes. In the early post-operative phase, a hyper-secretory phenotype in LPS-stimulated monocytes is associated with later development of pneumonia. Together, these findings suggest that innate immune status may influence the pathogenesis of post-operative pulmonary inflammation and could potentially aid stratification of post-operative risk in the future. Further dissection of the innate immune response may ultimately suggest plausible targets for the prevention of post-operative pneumonia. Finally, these data add to the case that VATS lobectomy should be the surgical treatment strategy of choice in early stage lung cancer.

Future Research

This study has linked post-operative pneumonia with host innate immune function, specifically pre-operative leucocyte counts in blood and BALF and early post-operative monocyte responses to LPS. These findings warrant further investigation in a larger cohort of patients across a range of centres. Determining the mechanisms through which these factors are linked to post-operative pneumonia, particularly monocyte hyper-responsiveness, would be important to target prophylactic/therapeutic measures. Ultimately, randomisation of prophylactic/therapeutic measures such as more prolonged courses of peri-operative antibiotics or immune modulators such as steroids in those patients stratified as high-risk may reduce the incidence of post-operative pneumonia.

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My personal role in the study involved contributing to the overall study design together with the establishment of individual experiment protocols. Monocyte subset analysis in particular was not included in the original grant application neither were the inclusion of pulmonary resections other than lobectomy. I obtained ethical approval to include all types of pulmonary resection as well as approval for recruitment of a control population to compare baseline immune measurements. I sourced the exhaled breath nitric oxide analysers and wrote the report to obtain university approval for procurement. I recruited and consented all patients, including the controls, to the study. I collected all samples and independently performed all laboratory experiments. The project also allowed me to develop flexible bronchoscopy and bronchoalveolar lavage skills, either directly performing these or assisting the surgeon with the protocol. I personally collected and analysed all the clinical data. I independently undertook all the analysis under the advice of the statistician except for the ANOVA modelling and calculation of the predictive strength of the biomarkers. I independently wrote the thesis, progress and final reports to funding body, The Chief Scientist Office.

Appendix

Abstract presented at The Society for Cardiothoracic Surgeons of Great Britain and Ireland, Annual Meeting, Brighton, March 2013. Awarded the Society Thoracic Medal.

Jones RO, Brittan M, Anderson NH, Conway Morris A, Murchison JT, Simpson AJ, Walker WS. Cellular Innate Immune Responses to Lung Resection Predict Post-Operative Pneumonia.

OBJECTIVE: Post-operative pneumonia is an important cause of morbidity and mortality following lung cancer resection. We hypothesise that early changes in innate immune parameters may help identify patients at greatest post-operative risk and potentially identify future therapeutic strategies.

METHODS: Patients undergoing lung resection for suspected bronchogenic carcinoma were recruited. Blood was sampled pre-operatively and at 6, 24 and 48 hours post-operatively. Neutrophil phagocytic capacity, monocyte subset changes and cytokine release from lipopolysaccharide (LPS)-stimulated monocytes were quantified in all patients at all time points. Bronchoalveolar lavage (BAL) was performed in the contralateral lung pre- and post-operatively. Screening for pneumonia was performed 72-hours post-operatively.

RESULTS: 40 patients were studied of which 14 (35%) developed post-operative pneumonia. Pre-operatively, a blood neutrophil count above 5.04×10^9/L had a relative risk (RR) for pneumonia of 3.3 (95% confidence interval (CI_{95}) 1.1-10.1), and a BAL cell count of greater than 1.04×10^5/ml had a RR of 3.4 (CI_{95} 1.3-9.0), whilst LPS-stimulated monocyte secretion of IL-12 of less than 0.15 pg/ml/μg protein had a RR of 3.0 (CI_{95} 1.2-7.3). At 24 hours post-operatively, LPS-stimulated release from

monocytes of IL-10 greater than 1.99 pg/ml/μg protein (RR 4.1, CI_{95} 1.3-12.3) and IL-6 greater than 414 pg/ml/μg protein (RR 3.1, CI_{95} 1.2-8.1) were predictive of pneumonia.

CONCLUSION: Relative pre-operative leucocytosis and relatively brisk monocyte responsiveness to stimulation in the early post-operative period are associated with development of pneumonia. These findings suggest novel markers for post-operative pneumonia and a role for monocytes in its pathogenesis.

Society for Cardiothoracic Surgery in Great Britain & Ireland

2nd April 2013

Mr R Jones

By email

Dear Mr Jones

Further to your recent presentation at our annual meeting in Brighton, I am writing to congratulate you on your achievement in winning the Society Thoracic medal for your presentation entitled **Cellular Innate Immune Responses to Lung Resection Predict Post-Operative Pneumonia.**

In recognition of your achievement, it is our pleasure to invite you on behalf of the President and Executive Committee to attend next year's annual dinner in Edinburgh where you will be officially presented with your prize. I will send you further details nearer the time

If you have any questions, please do not hesitate to contact me using the details below.

Yours sincerely

Isabelle Ferner
Society Administrator &
Conference Organiser

Abstract presented at 26th EACTS Annual Meeting, Barcelona, Spain, 29th October 2012.

Jones RO, Anderson NH, Murchison JT, Brittan M, Simon EJ, Casali G, Simpson AJ, Walker WS. Innate Immune Function Following Major Lung Resection For Bronchogenic Carcinoma via Video-Assisted Thoracoscopic Surgery and Thoracotomy. **Interactive Cardiovascular and Thoracic Surgery** 2012; 15 (2): S82.

Objectives: To prospectively compare the innate immune response of patients undergoing lung resection for bronchogenic carcinoma via Video-Assisted Thoracoscopic Surgery (VATS) and thoracotomy. Improved understanding of peri-operative inflammatory responses may help elucidate immediate post-operative complications such as pneumonia and ALI/ARDS and may ultimately lead to improvements in patient survival.

Methods: Lung resection for suspected bronchogenic carcinoma was performed with a VATS approach or thoracotomy. Bronchoalveolar lavage (BAL) was conducted in the contra-lateral lung prior to staging bronchoscopy and mediastinoscopy and immediately after lung resection. Blood and exhaled nitric oxide was sampled pre-operatively and at 6-, 24- and 48-hours post-operatively.

Results: Forty patients were included in the study (26 VATS, 14 thoracotomy). There was a lower systemic cytokine response from VATS patients compared with thoracotomy for serum IL-6 (ANOVA p=0.026), IL-8 (ANOVA p=0.018) and IL-10 (ANOVA p=0.047). A trend for lower post-operative blood neutrophil counts in VATS patients fell just short of significant (ANOVA p=0.054). VATS surgery resulted in a lower fall in serum albumin post-operatively (ANOVA p=0.001). Lower levels of IL-10 were produced by lipopolysaccharide (LPS)-stimulated blood monocytes from VATS patients compared with thoracotomy at 6 hrs post-operatively (geometric mean ratio

1.16 95% CI 1.08-1.33 p=0.011). No statistically significant difference in the neutrophil phagocytic capacity, lymphocyte count, monocyte count or monocyte subset count was found between the surgical groups (ANOVA p>0.05). No statistically significant differences in BAL fluid parameters were found. Exhaled nitric oxide levels fell post-operatively which reached statistical significance at 48hrs (geometric mean ratio 1.2 95% CI 1.02-1.46 p=0.029). There was no significant difference found between patients undergoing resection via VATS or thoracotomy (ANOVA p=0.331).

Conclusion: Greater pro- and anti-inflammatory responses are seen with lung resection performed via thoracotomy compared with VATS. These data further support increased use of VATS lobectomy techniques.

Abstract presented at The British Thoracic Society (BTS), Winter Meeting, London, 6 December 2012.

Jones RO, Murchison JT, Casali G, Simon EJ, Anderson N, Simpson AJ, Walker WS. Bronchoalveolar Lavage Does Not Affect The Acute Inflammatory Response Following Bronchoscopy and Mediastinoscopy**. Thorax** 2012; 67; (Suppl 2): A110.

Objective: Bronchoalveolar lavage (BAL) may be used to investigate acute inflammation following thoracic surgery. However, BAL has previously been found to induce an acute phase response in healthy and critically-ill patients complicating research analysis (1,2). We sought to examine the impact of BAL in thoracic surgery patients hypothesising that BAL would not lead to a significant additional acute inflammatory response.

Methods: Seventeen patients undergoing lung cancer staging bronchoscopy and mediastinoscopy were randomly assigned to have 220mls 0.9% NaCl BAL before surgery (n=10) or no BAL (n=7). Blood samples were taken pre-operatively followed by 6 and 24 hours post-operatively. Exhaled nitric oxide (eNO) was also measured at a flow rate of 50mls/sec at these times. All patients had a CXR at 24hrs and were evaluated for evidence of SIRS using pre-defined criteria.

Results: IL-6 and CRP increased post-operatively peaking at 6hrs and 24hrs respectively however there was no statistically significant difference between the increase for BAL and non-BAL patients ($p>0.05$). There was no significant increase or variation between the groups for IL-2, IL-4, IL-10, TNF-α or IFN-γ ($p>0.05$). eNO tended to decrease in the BAL group and increase in the non-BAL group at 6hrs although there was no significant difference between the groups ($p=0.167$). Post-operative CXR atelectasis developed in 3 patients (2 BAL). One patient in each group developed SIRS.

Conclusion: BAL has minimal impact on acute inflammation following bronchoscopy and mediastinoscopy. It may therefore be used to safely and reliably obtain samples for research or microbiology purposes in thoracic surgery patients.

1. Huang Y-C T, Bassett MA, Levin D, Montilla, Ghio AJ. Acute Phase Reaction in Healthy Volunteers After Bronchoscopy with Lavage. Chest 2006; 129 (6): 1565-9.
2. Terashima T, Amakawa K, Matsumaru A et al. BAL Induces an Increase in Peripheral Blood Neutrophils and Cytokine Levels in Healthy Volunteers and Patients with Pneumonia. Chest 2001; 119 (6): 1724-1729.